**PCT** ORLD INTELLECTUAL PROPERTY ORGANIZATIC..
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| (51) International Patent Classification 5 : | | (11) International Publication Number: WO 93/00426 |
|---|---|---|
| C12N 9/20, 15/55 // C12R 1:69 | A1 | (43) International Publication Date: 7 January 1993 (07.01.93) |

(21) International Application Number: PCT/DK92/00200

(22) International Filing Date: 25 June 1992 (25.06.92)

(30) Priority data:
91610056.3 25 June 1991 (25.06.91) EP
*(34) Countries for which the regional or international application was filed:* DK et al.

(71) Applicant *(for all designated States except US):* NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only) :* HJORTH, Siv, Annegrethe [DK/DK]; Norgesvej 18, A, DK-2800 Lyngby (DK). WOLDIKE, Helle, Fabricius [DK/DK]; Stendyssevej 12, DK-3540 Lynge (DK). THIM, Lars [DK/DK]; Skiftevej 22, DK-2820 Gentofte (DK). GORMSEN, Erik [DK/DK]; Snekketoften 15, DK-2830 Virum (DK). VERGER, Robert [FR/FR]; 230, avenue de la Panouse, F-13009 Marseille (FR).

(74) Common Representative: NOVO NORDISK A/S; Novo Allé, DK-2880 Bagsværd (DK).

(81) Designated States: BR, CA, FI, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: MAMMALIAN PANCREATIC LIPASE AND VARIANT THEREOF

(57) Abstract

A guinea pig lipase (GPL) essentially free from other guinea pig proteins which has the amino acid sequence shown in the appended Sequence Listing ID No. 2 or an enzymatically active variant thereof as well as certain variants of the Human Pancreatic Lipase (HPL). The GPL and GPL and HPL variants may be prepared by recombinant DNA techniques and may be used as constituents in a detergent composition or as a digestive enzyme.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|---|---|---|---|---|---|
| **AT** | Austria | **FI** | Finland | **ML** | Mali |
| **AU** | Australia | **FR** | France | **MN** | Mongolia |
| **BB** | Barbados | **GA** | Gabon | **MR** | Mauritania |
| **BE** | Belgium | **GB** | United Kingdom | **MW** | Malawi |
| **BF** | Burkina Faso | **GN** | Guinea | **NL** | Netherlands |
| **BG** | Bulgaria | **GR** | Greece | **NO** | Norway |
| **BJ** | Benin | **HU** | Hungary | **PL** | Poland |
| **BR** | Brazil | **IE** | Ireland | **RO** | Romania |
| **CA** | Canada | **IT** | Italy | **RU** | Russian Federation |
| **CF** | Central African Republic | **JP** | Japan | **SD** | Sudan |
| **CG** | Congo | **KP** | Democratic People's Republic of Korea | **SE** | Sweden |
| **CH** | Switzerland | | | **SN** | Senegal |
| **CI** | Côte d'Ivoire | **KR** | Republic of Korea | **SU** | Soviet Union |
| **CM** | Cameroon | **LI** | Liechtenstein | **TD** | Chad |
| **CS** | Czechoslovakia | **LK** | Sri Lanka | **TG** | Togo |
| **DE** | Germany | **LU** | Luxembourg | **US** | United States of America |
| **DK** | Denmark | **MC** | Monaco | | |
| **ES** | Spain | **MG** | Madagascar | | |

# MAMMALIAN PANCREATIC LIPASE AND VARIANT THEREOF

## FIELD OF INVENTION

The present invention relates to mammalian pancreatic lipase and variants thereof, a DNA sequence encoding the lipase, a method of producing mammalian pancreatic lipases and lipase variants, and their use as detergent enzymes and digestive enzymes.

## BACKGROUND OF THE INVENTION

The DNA sequence encoding human pancreatic lipase, and the tertiary structure of the enzyme appears from F.K. Winkler et al., Nature 343, 1990, pp. 771-774. One of the outstanding features of the enzyme is the presence of a surface loop structure between Cys 238 and Cys 262 which covers the active site when the lipase is in inactive form, and which changes its conformation when the lipase is activated so as to make the active serine accessible to a lipid substrate (cf. also Brady et al., Nature 343, 1990, pp. 767-770, describing a similar structure of a microbial lipase).

The purification of lipase from guinea pig pancreas is described by J. Fauvel et al., Biochim. Biophys. Acta 663, 1981, pp. 446-456. It is noted that, apart from lipase activity, guinea pig pancreatic lipase also has phospholipase activity.

## DESCRIPTION OF THE INVENTION

Guinea pig pancreatic lipase has now been cloned and sequenced at the DNA and amino acid level. Although the guinea pig lipase has a high degree of sequence homology to the human pancreatic enzyme, it was surprisingly found that the guinea pig lipase lacks a loop structure corresponding to that covering the active site of the human lipase. It is therefore envisaged that the guinea pig lipase has certain properties which differ from

those of the human lipase such as a different substrate specificity.

Accordingly, in one aspect, the present invention relates to a guinea pig pancreatic lipase (GPL) essentially free from other guinea pig proteins which has the amino acid sequence shown in the appended Sequence Listing ID No. 2, or an enzymatically active variant thereof.

In the present context, the term "variant" is intended to indicate a polypeptide which is derived from the native lipase protein by suitably modifying the DNA sequence coding for the native lipase, resulting in the addition of one or more amino acid residues to either or both the N- and C-terminal end of the native enzyme, substitution of one or more amino acid residues at one or more different sites in the amino acid sequence, deletion of one or more amino acid residues at either or both ends of the native protein or at one or more sites in the amino acid sequence, or insertion of one or more amino acid residues at one or more sites in the amino acid sequence. The term "variant" is also intended to include naturally occurring variants of the enzyme which are homologous to the amino acid sequence shown herein, e.g. pancreatic lipases of other mammalian species such as porcine, bovine, canine, feline or coypu pancreatic lipase, or enzymatically active derivatives thereof.

The term "enzymatically active" as used about the GPL variant is intended to indicate that the variant should have at least one enzymatic activity and normally more, especially one or more of the enzymatic activities of the GPL. It has been found that, apart from lipase and phospholipase activity, the native GPL also exhibits esterase activity and presumably protease activity. Any of the enzymatic activities of the GPL, and thus of a GPL variant as defined herein, may be determined using assays known in the art for the enzymatic activity in question.

The region of the enzyme which gives rise to at least some of the esterase activity is presumed to be localized to the C-terminal end of the lipase (cf. J.D. De Caro et al., Eur. J. Biochem. 158, 1987, pp. 601-607). Thus, if one wishes to prepare a smaller GPL molecule with decreased esterase activity, but retained lipase activity, GPL may be C-terminally truncated. A preferred GPL variant of this type is $GPL_{1-319}$.

As indicated above, it has surprisingly been found that GPL lacks the loop structure which, in HPL, covers the active site of the enzyme when the HPL is in the inactive state. More specifically, the GPL of the invention has a sequence of five amino acids (Lys-Thr-Gly-Ile-Ser) between the amino acids 239 and 245 rather than the longer loop structure known from HPL. It may therefore be possible to prepare a GPL variant with different enzymatic properties than the native enzyme, said variant comprising an insertional substitution between amino acid 239 and amino acid 245 of the native GPL sequence. In particular, the insertional substitution comprises the following amino acid sequence

```
Lys          Ala                                  Asn Leu
Gln Lys Asn Ile Leu Ser Gln Ile Val Asp Ile Asp Gly Ile Trp Glu

                        Ala
Gly Thr Arg Phe Val Ala
```

or a fragment thereof capable of functioning as a loop structure covering the active site in the inactive state of the enzyme. In a preferred embodiment, the insertional substitution comprises the amino acid sequence

Lys Lys Asn Ile Leu Ser Gln Ile Val Asp Ile Asp Gly Ile Trp Glu
Gly Thr Arg Asp Phe Ala Ala

(corresponding to the loop structure found in HPL). The construction of this GPL variant is described in Example 6.

Furthermore, it has been found that either $Cys_{103}$ or $Cys_{105}$ of native GPL may be non-disulfide linked. The free -SH group present on the cystine residue is sensitive to oxidation, which may be detrimental to the storage stability of the enzyme or its performance as a detergent enzyme. It may therefore be an advantage to provide a GPL variant, wherein $Cys_{103}$ or $Cys_{105}$ is replaced by another amino acid residue. Such an amino acid residue is preferably an uncharged and non-bulky residue, and may preferably be selected from Ser or Thr.

A still further GPL variant of the invention is one in which an amino acid of the catalytic triad Ser-His-Asp is replaced by another residue, in particular in which $Ser_{154}$ is replaced by Thr and/or $Asp_{178}$ is replaced by Glu.

It should be noted that, according to the invention, any one of the modifications of the native GPL protein described above may be combined with one or more of the other modifications, resulting in a GPL variant which exhibits two or more of the features noted above.

In another aspect, the present invention relates to a recombinant DNA molecule comprising a DNA sequence encoding GPL, or an enzymatically active variant thereof. In particular, the invention relates to a recombinant DNA molecule with the DNA sequence shown in the appended Sequence Listing ID No. 1, or a modification of said sequence encoding GPL or an enzymatically active variant thereof.

Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another amino acid sequence of the lipase, but which may correspond to the codon usage of the host organism into which the recombinant DNA molecule is introduced (i.e. modifications which, when expressed, results in GPL with the amino acid sequence shown in the appended Sequence Listing ID no. 2), or nucleotide substitutions which do give rise to a different amino acid sequence and therefore, possibly, a different polypeptide

structure without, however, impairing the properties of the lipase (i.e. modifications which, when expressed, results in enzymatically active GPL variants as defined herein). Other examples of possible modifications are insertion of one or more nucleotides into the sequence, addition of one or more nucleotides at either end of the sequence and deletion of one or more nucleotides at either end of or within the sequence. Specific modifications of the DNA sequence are those which, when expressed, give rise to any one of the GPL variants described above.

A DNA sequence encoding the present enzyme or an enzymatically active variant thereof may, for instance, be isolated by establishing a guinea pig cDNA or genomic library and screening for positive clones by conventional procedures such as by hybridization to oligonucleotide probes synthesized on the basis of the full or partial amino acid sequence of the enzyme or by selecting for clones expressing the appropriate enzyme activity, or by selecting for clones producing a protein which is reactive with an antibody raised against the native GPL. In this case, a genomic or cDNA sequence encoding the lipase may be modified at a site corresponding to the site(s) at which it is desired to introduce amino acid substitutions, e.g. by site-directed mutagenesis using synthetic oligonucleotides encoding the desired amino acid sequence for homologous recombination in accordance with well-known procedures.

Alternatively, the DNA sequence encoding the enzyme or an enzymatically active variant thereof may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., The EMBO J. 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in appropriate vectors.

Finally, the DNA sequence may be of mixed genomic and synthetic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to various parts of the entire recombinant DNA molecule, in accordance with standard techniques. The recombinant DNA molecule may also be prepared by polymerase chain reaction using specific primers, for instance as described in US 4,683,202 or R.K. Saiki et al., Science 239, 1988, pp. 487-491.

In a further aspect, the present invention relates to certain variants of human pancreatic lipase (HPL). Thus, in one embodiment, the invention relates to a HPL variant which is deleted of one or more amino acids in the sequence between $Cys_{238}$ and $Cys_{262}$, in other words, in which the loop structure described by Winkler et al., op. cit. has been altered. For example, the entire sequence constituting the loop structure may be deleted or replaced by the sequence Lys-Thr-Gly-Ile-Ser (i.e. the corresponding "loop" sequence of GPL). In Example 5 the construction of a HPL variant with no loop structure is described.

In another embodiment, the HPL variant may be one which is C-terminally truncated for the preparation of a HPL variant with decreased esterase activity, as noted above for the C-terminally truncated GPL variant. A particularly preferred variant of this type is $HPL_{1-336}$.

Similarly to GPL, native HPL contains a non-disulfide linked cystine residue at either $Cys_{102}$ or $Cys_{104}$. The free -SH group present on the cystine residue is sensitive to oxidation, which may be detrimental to the storage stability of the enzyme or its performance as a detergent enzyme. It may therefore be an advantage to provide a HPL variant, wherein $Cys_{102}$ or $Cys_{104}$ is replaced by another amino acid residue. Such an amino acid residue is preferably an uncharged and non-bulky residue, and may preferably be selected from Ser or Thr.

A still further HPL variant of the invention is one in which an amino acid of the catalytic triad Ser-His-Asp is replaced by another residue, in particular in which $Ser_{153}$ is replaced by Thr and/or $Asp_{177}$ is replaced by Glu.

It should be noted that, according to the invention, any one of the modifications of the native HPL protein described above may be combined with one or more of the other modifications, resulting in a HPL variant which exhibits two or more of the features noted above.

Another type of lipase variants of the present invention is an enzymatically active recombinant lipase which comprises at least one GPL fragment and at least one HPL fragment, i.e. a hybrid lipase. These recombinant lipase variants are expected to provide interesting new activity profiles combining some of the enzymatic properties exerted by HPL and GPL, respectively, in their native state. In the present context the terms "GPL fragment" and "HPL fragment" are intended to indicate any fragment of GPL and HPL, respectively, which is sufficiently large to be recognized as such, for instance a fragment comprising at least 10 amino acid residues. The recombinant variants may be prepared by in vivo or in vitro recombination, e.g. as explained in Example 12 and 13.

In a further aspect the present invention relates to a DNA sequence encoding any of the lipase variants disclosed herein. A DNA sequence encoding a recombinant lipase variant as described above may be prepared by in vitro or in vivo recombination as described in Examples 12 and 13 from DNA encoding GPL or HPL or variants thereof. Furthermore, the DNA sequence encoding a recombinant lipase variant may be prepared synthetically, e.g. as described above.

The GPL and GPL and HPL variants of the invention are suitably prepared on the basis of recombinant DNA procedures known in the art using eukaryotic or prokaryotic expression systems, examples of which are a mammalian expression system, an insect

expression system, a fungal (including yeast) expression system and an bacterial expression system. For this purpose, a suitable host cell harbouring a recombinant expression vector carrying a DNA sequence encoding the enzyme is cultured under conditions conducive to the production of the enzyme.

The recombinant expression vector into which the DNA sequence is inserted may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the enzyme should be operably connected to a suitable promoter sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. Examples of suitable promoters for directing the transcription of the DNA encoding the enzyme in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814) or the adenovirus 2 major late promoter. Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4, 599, 311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Promoters suitable for use in filamentous fungi are described below.

The DNA sequence encoding the GPL or a GPL or HPL variant of the invention may also be operably connected to a suitable terminator, such as the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 terminator (Alber and Kawasaki, op. cit.). The vector may further comprise elements such as polyadenylation signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

The recombinant expression vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An examples of such a sequence (when the host cell is a mammalian cell) is the SV 40 origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or one which confers resistance to a drug, e.g. neomycin, hygromycin or methotrexate.

The procedures used to ligate the DNA sequences coding for the mammalian pancreatic lipase, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989).

The host cell into which the expression vector is introduced may be any cell which is capable of producing the mammalian pancreatic lipase an example of which is a eukaryotic cell, in particular a mammalian cell. Methods of transfecting mammalian cells and expressing DNA sequences introduced in the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159, 1982, pp. 601-621; Southern and Berg, J. Mol. Appl. Genet. 1, 1982, pp. 327-341; Loyter et al., Proc. Natl. Acad. Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and Pearson, Somatic Cell Genetics 7, 1981, p. 603,

Graham and van der Eb, Virology 52, 1973, p. 456; and Neumann et al., EMBO J. 1, 1982, pp. 841-845.

Also insect cells may be used as host cells for the expression of the mammalian pancreatic lipase, for instance, using the principles described by Summers, M.D. and Smith, G.E., 1987, A Manual of Methods for Baculovirus Vectors and the Insect Cell Culture Procedures, Texas Agricultural Experiment Station & Texas A&M University, College Station Texas 77843-2475).

Alternatively, fungal cells (including yeast cells) or bacterial cells may be used as host cells. Examples of suitable yeast cells include cells of Saccharomyces spp. or Schizosaccharomyces spp., in particular strains of Saccharomyces cerevisiae. Examples of bacterial cells include cells of the genus Bacillus, especially B. subtilis. An example of suitable fungal cells, i.e. a filamentous fungus, will be dealt with in detail below.

The medium used to culture the cells may be any conventional medium suitable for growing the cells in question, such as a serum-containing or serum-free medium containing appropriate supplements (for mammalian cells). Suitable media are available from commercial suppliers or may be prepared according to published recipes (e.g. in catalogues of the American Type Culture Collection).

While intracellular expression may be advantageous in some respects, e.g. when using certain bacteria as host cells, it is generally preferred that the expression is extracellular. In order to obtain extracellular expression, the expression vector should normally further comprise a DNA sequence encoding a preregion permitting secretion of the expressed mammalian pancreatic lipase into the culture medium.

The mammalian pancreatic lipase may be recovered from the medium by conventional procedures including separating the host cells from the medium by centrifugation or filtration,

precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulphate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography, affinity chromatography, or the like.

While any of the above explained expression systems may be suitable for the production of mammalian pancreatic lipases the presently preferred process for the production of a mammalian pancreatic lipase is by use of a filamentous fungus as a host cell. Accordingly, in a still further aspect, the present invention relates to a process for the extracellular production of a mammalian pancreatic lipase in a filamentous fungus, the process comprising

(a) providing a recombinant DNA vector which comprises a DNA sequence encoding a mammalian pancreatic lipase, and a DNA sequence encoding a preregion permitting secretion of the expressed mammalian pancreatic lipase into the culture medium,

(b) transforming a suitable filamentous fungus with the recombinant DNA vector of step (a), and

(c) culturing the transformed filamentous fungus in a suitable culture medium under conditions conducive to the production of the mammalian pancreatic lipase.

The term "filamentous fungus" is intended to include fungi belonging to the groups Phycomycetes, Zygomycetes, Ascomycetes, Basidiomycetes or fungi imperfecti, including Hyphomycetes such as the genera Aspergillus, Trichoderma, Penicillium, Fusarium or Humicola.

It is evident that the recombinant DNA vector further comprises DNA sequences encoding functions permitting gene expression, and where appropriate a DNA sequence coding for a suitable marker for the selection of transformants.

DNA sequences encoding functions permitting gene expression typically comprise a promoter, transcription initiation sites, and transcription termination and polyadenylation functions.

The promoter which may be preceded by upstream activating sequences and enhancer sequences as known in the art may be any DNA sequence exhibiting a strong transcriptional activity in filamentous fungi and may be derived from a gene encoding an extracellular or intracellular protein such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.

Examples of suitable promoters are those derived from the gene encoding A. oryzae TAKA amylase, Rhizomucor miehei aspartic proteinase, A. niger neutral α-amylase, A. niger acid stable α-amylase, A. niger glucoamylase, Rhizomucor miehei lipase, A. oryzae alkaline protease or A. oryzae triose phosphate isomerase.

The filamentous fungus host organism may conveniently be one which has previously been used as a host for producing recombinant proteins, e.g. a strain of Aspergillus sp., such as A. niger, A. nidulans or A. oryzae. The use of A. oryzae in the production of recombinant proteins is extensively described in, e.g. EP 238 023.

In particular when the host organism is A. oryzae, a preferred promoter for use in the process of the present invention is the A. oryzae TAKA amylase promoter as it exhibits a strong transcriptional activity in A. oryzae. The sequence of the TAKA amylase promoter appears from EP 238 023.

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The techniques used to transform a fungal host cell may suitably be adapted from the methods of transforming A. nidulans described in, for instance, Yelton et al., Proc. Natl.

Acad. Sci. USA 81, 1984, pp. 1470-1474, or EP 215 594, or from the methods of transforming A. niger described in, for instance Buxton et al., Gene 37, 1985, pp. 207-215 or US 4,885,249, or from the method of transforming A. oryzae described in EP 238 023. In the process of the present invention, the host cell may be transformed with a vector comprising a DNA sequence coding for a selection marker which is capable of being incorporated in the genome of the host organism on transformation, but which is either not expressed by the host before transformation or expressed in amounts which are not sufficient to permit growth under selective conditions. Transformants can then be selected and isolated from non-transformants on the basis of the incorporated selection marker.

Suitable selection markers are derived from the A. nidulans or A. niger argB gene, the A. nidulans trpC gene, the A. nidulans amdS gene, the Neurospora crassa pyr4 or DHFR genes, or the A. niger or A. oryzae niaD gene.

Preferred selection markers for use in the present invention are derived from the A. nidulans or A. niger amdS or argB genes. Wild-type A. oryzae strains are usually $ArgB^+$ (which means that the argB gene is expressed in A. oryzae). Thus, if argB is chosen as the selection marker, an $ArgB^-$ mutant strain of A. oryzae (which does not express the ArgB gene) must be used as the host organism. On the other hand, the amdS gene may be used as the selection marker in wild-type A. oryzae strains which do not express this gene in sufficient amounts to permit growth under selective conditions.

The preregion provided on the vector to ensure efficient direction of the expressed product into the secretory pathway of the host cell may be a naturally occurring signal or leader peptide or a functional part thereof or a synthetic sequence providing secretion of the protein from the cell. Thus, the preregion may be derived from a gene coding for a secreted protein derived from any source.

In particular, the preregion may be derived from a gene encoding an Aspergillus sp. amylase or glucoamylase, a gene encoding a Rhizomucor miehei lipase or protease, a gene encoding a Coprinus sp. peroxidase, a gene encoding a Humicola cellulase or xylanase, or a gene encoding a mammalian pancreatic lipase.

The preregion is preferably derived from the gene encoding A. oryzae TAKA amylase, A. niger neutral α-amylase, A. niger acid-stable α-amylase, A. niger glucoamylase, or a Coprinus macrorhizus or cinereus peroxidase, or H. insolens cellulase or xylanase, or the gene encoding guinea pig, human, canine or porcine pancreatic lipase.

The DNA sequences coding for the lipase, preregion, promoter and terminator may be inserted in a vector containing the selection marker, or it may be inserted in a separate vector for introduction into the host cell. The vector or vectors may be linear or closed circular molecules. In a preferred embodiment of the process of the invention, two vectors are used, one carrying the DNA sequence coding for the selection marker, and the other carrying the DNA sequences encoding the lipase, the preregion and the functions permitting gene expression.

The mammalian pancreatic lipase producible by the process of the invention may, in principle, be derived from any mammalian source, and may thus for instance be of bovine, porcine, canine, coypu, human or guinea pig origin. Guinea pig lipase may, in particular, be one encoded by the recombinant DNA molecule of the present invention, as described above. Human pancreatic lipase is preferably a HPL variant according to the invention, as described above.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing filamentous fungi. The transformants are usually stable and may be cultured in the absence of selection pressure. However, if the transformants

are found to be unstable, the selection marker introduced into the cells may be used for selection.

The mature lipase protein secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures including separating the cells from the medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

The present invention also relates to a detergent additive comprising GPL or a GPL or a HPL variant according to the invention, preferably in the form of a non-dusting granulate, stabilized liquid or protected enzyme. Non-dusting granulates may be produced e.g. according to US 4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent additive may suitably contain 0.2-200 mg of enzyme protein per gram of the additive. It will be understood that the detergent additive may further include one or more other enzymes, such as a protease, cellulase, peroxidase or amylase, conventionally included in detergent additives.

In a still further aspect, the invention relates to a detergent composition comprising GPL or a GPL or a HPL variant of the invention. Detergent compositions of the invention additionally comprise surfactants which may be of the anionic, non-ionic, cationic, amphoteric, or zwitterionic type as well as mixtures of these surfactant classes. Typical examples of anionic surfactants are linear alkyl benzene sulfonates (LAS), alpha olefin sulfonates (AOS), alkyl sulphate (AS), alcohol ethoxy

sulfates (AES), alcohol ethoxylates (AEO) and alkali metal salts of natural fatty acids. However, according to the invention, it has been found most advantageous for the stability and activity of the enzyme to include an alkyl phosphate or AEO or a combination thereof as the surfactant.

Detergent compositions of the invention may contain other detergent ingredients known in the art as e.g. builders, bleaching agents, bleach activators, anti-corrosion agents, sequestering agents, anti soil-redeposition agents, perfumes, enzyme stabilizers, etc.

The detergent composition of the invention may be formulated in any convenient form, e.g. as a powder or liquid. The enzyme may be stabilized in a liquid detergent by inclusion of enzyme stabilizers as indicated above. Usually, the pH of a solution of the detergent composition of the invention will be 7-12 and in some instances 7.0-10.5. Other detergent enzymes such as other lipases, proteases, cellulases or amylases may be included the detergent compositions of the invention, either separately or in a combined additive as described above.

The present invention further relates to a GPL or a HPL variant of the invention for use as a digestive enzyme. The oral substitution of pancreatic lipase is of vital importance in the treatment of patients suffering from severe exocrine pancreatic insufficiency, such as cystic fibrosis and chronic pancreatitis, which leads to malabsorption and steatorrhoea. Conventional enzyme preparations for this purpose are mainly based on enzyme extracts of porcine pancreas and also contains other enzymes, primarily proteases and amylases. However, it has been found that the content of lipase in these preparations is too low to ensure an adequate enzymatic degradation of triglycerides in the diet so as to result in a normal lipid absorption from the gastrointestinal tract. It has previously been suggested to use microbial lipases as digestive aids (DE 16 42 654 and GB 1 442 677). The use of recombinant human gastric lipase for treating lipase deficiency is suggested in

WO 86/01532. It is, however, assumed to be an advantage to use pancreatic lipase as a digestive aid in case of patients with pancreatic insufficiency as these patients produce normal levels of gastric lipase and as the pancreatic lipase is active in an intestinal rather than gastric environment.

The present invention is further illustrated in the appended figures in which

Fig. 1 illustrates the construction of the human pancreatic lipase mutant (HPL(-)) with no loop structure further explained in Example 5,

Fig. 2 illustrates the construction of the guinea pig pancreatic lipase mutant (GPL(+)) containing a loop structure further explained in Example 6,

Fig. 3 illustrates the oligonucleotide sequences used for the construction of the truncated guinea pig pancreatic lipase ($GPL_{1-319}$) further explained in Example 7,

Fig. 4 illustrates the construction of the expression plasmid harbouring the guinea pig pancreatic lipase gene which is described in Example 8,

Fig. 5 is a graph illustrating the activity of recombinant guinea pig lipase as compared to that of a H.lanuginosa lipase in the olive oil assay further described in Example 11,

Figs. 6 and 7 illustrates the pH and temperature profile, respectively, of the recombinant guinea pig lipase in a tributyrin assay further discussed in Example 11,

Fig. 8 illustrates the activity of recombinant guinea pig lipase in the presence of the surfactants listed in Example 11, as determined in the olive oil assay, and

Fig. 9 illustrates the procedure used for constructing the in vitro recombined hybrids described in Example 13.

The present invention is further illustrated in the following examples which are not intended to limit, in any way, the scope of the invention as claimed.

**Example 1**

**Purification and partial sequencing of native GPL**

Guinea pig pancreatic lipase (GPL) was purified to homogeneity essentially as previously described (Fauvel J. et al. Biochim. Biophys. Acta 663 (1981) 446-456). Approx. 0.6 nmol of purified GPL was subjected to automatic Edman degradation using an Applied Biosystems 470 A gas phase sequencer as previously described (Thim, L. et al. FEBS Lett. 217 (1987) 307-312. The result from this sequence analysis is given in Table 1. Approx. 1.4 nmol of GPL was dissolved in 100 µl of 0.2M TRIS · HCl buffer, Ph = 7.5 and 57 mg guanidine hydrochloride was added. The lipase was reduced by the addition of 0.25 mg dithiothreitol and incubation at room temperature for 16 h. The resulting protein was derivatized by the addition of 1 µl 4-vinyl pyridine and incubation at room temperature for 1 h. The reaction was stopped by the addition of 1.0 mg dithiothreitol and the pyridyl ethylated GPL (PE-GPL) was purified by reverse phase HPLC on a Vydac C-4, 4.6 x 250 mm, column. The column was equilibrated and eluted with a linear gradient mixture of acetonitrile in 0.1% trifluoroacetic acid. PE-GPL eluted at approx. 44% of acetonitrile.

The purified PE-GPL was lyophilized and redissolved in 200 µl of 50 mM sodium phosphate buffer, pH = 8.0 and digested by the addition of 0.5 µg trypsin and incubation at 37°C for 24 h. The protein digest was submitted to peptide mapping using the HPLC-system described above. Peptide fragments were isolated and subjected to amino acid sequence analysis as described above.

The sequencing results of 3 of these fragments are given in Table 2.

Table 1

Amino acid sequence analysis of native GPL

| Cycle No. | PTH-a.a. | Yield (pmol) |
|---|---|---|
| 1 | Ala | 342 |
| 2 | Glu | 127 |
| 3 | Val | 183 |
| 4 | PE-Cys | 35 |
| 5 | Tyr | 114 |
| 6 | Ser | 64 |
| 7 | His | 10 |
| 8 | Leu | 122 |
| 9 | Gly | 116 |
| 10 | PE-Cys | 49 |
| 11 | Phe | 89 |
| 12 | Ser | 49 |
| 13 | Asp | 89 |
| 14 | Glu | 63 |
| 15 | Lys | 67 |
| 16 | Pro | 81 |
| 17 | Trp | trace |
| 18 | Ala | 69 |
| 19 | Gly | 71 |
| 20 | Thr | 19 |

Table 2

Amino acid sequence analysis of tryptic fragment of E-GPL

| Cycle No. | Fragment No. 49 | | Fragment No. 22 | | Fragment No. 44 | |
|---|---|---|---|---|---|---|
| | PTH-a.a. | Yield (pmol) | PTH-a.a. | Yield (pmol) | PTH-a.a. | Yield (pmol) |
| 1 | Tyr | 16 | Met | 12 | Val | 28 |
| 2 | Asn | 27 | Gly | 12 | Thr | 21 |
| 3 | Phe | 23 | His | 10 | Phe | 12 |
| 4 | PE-Cys | 19 | Phe | 8 | Leu | 14 |
| 5 | Ser | 10 | Ala | 10 | Asp | 4 |
| 6 | Ser | 9 | Asp | 8 | Pro | 4 |
| 7 | Asp | 11 | Gln | 8 | Ala | 4 |
| 8 | Ile | 10 | Tyr | 7 | Glu | 3 |
| 9 | Val | 7 | Pro | 7 | Pro | 2 |
| 10 | Gln | 11 | Gly | 9 | | |
| 11 | Glu | 9 | Lys | 2 | | |
| 12 | Asn | 11 | | | | |
| 13 | Val | 4 | | | | |
| 14 | Glu | 9 | | | | |
| 15 | Gln | 8 | | | | |
| 16 | Thr | 5 | | | | |
| 17 | Leu | 4 | | | | |
| 18 | Ser | 3 | | | | |
| 19 | Pro | 3 | | | | |

Example 2

Construction of a cDNA library from guinea pig pancreas

Total RNA was extracted from homogenized guinea pig pancreas according to the procedure described by Chirgwin et al. (Biochemistry (1979) 18, 5294-5299). Messenger RNA was obtained by two cycles of affinity chromatography on oligo (dT) cellulose as described by Aviv and Leder ( Proc. Natl. Acad. Aci. USA (1972) 69, 1408-1412).

A cDNA library was constructed starting from 3.5 µg of mRNA using a slightly modified version of Okyama and Berg protocol (Okayama and Berg (1982) Mol. Cell. Biology 2, 161-170) eliminating poly(dA) purification of oligo(dT) tailed primer fragment.

The cloning vectors used were as follows:
The vector primer fragment was pCDV1-PL8 linearized by KpnI. The plasmid pCDV1-PL8 is a derivative of the pCDV1-PL plasmid as originally described by Noma et al (1986) Nature 319, 640-646. The modification of pCDVL-PL1 consists of insertion of a NotI site immediately adjacent to the KpnI thus permitting linearization of the plasmid 3′ of the cDNA insert. The linker fragment consisted of an oligo(dG)-tailed HindIII-SacI fragment of the pSP62-K2 plasmid (Noma et al (1986) Nature 319, 640-646).

The cDNA library was transformed into competent SCS1 cells (Stratagene) according to the high-transformation-efficiency procedure described by Hanahan et al (1983) J.Mol.Biol. 166, 557-580. Approximately 10.000 recombinant clones were obtained.

Example 3

Isolation and characterization of a cDNA clone encoding guinea pig pancreatic lipase (GPL):

Recombinant clones obtained as described in Example 2 were transferred to Millipore nitrocellulose filters and immobilized as described in "Molecular Cloning" Ed. Sambrook et al. (1989).

Hybridization probes were chosen according to the amino acid sequence data resulting from microsequencing (cf. Example 1) of two fragments of the purified guinea pig pancreatic lipase.

The oligonucleotides which were synthesized on an Applied Biosystems, Inc. DNA synthesizer were of the following sequence and composition:

#1853:

5'GA(TC) GA(AG) AA(AG) CC(ATGC) TGG GC3' (degeneracy=32)
corresponding to the sequence
Asp-Glu-Lys-Pro-Trp-(Ala) of the N-terminal fragment.

and

#1854:

5' Ca(AG) GA(AG) AA(TC) GT(ATGC) GA(AG) CA3'
(degeneracy=64)
corresponding to the sequence
Gln-Glu-Asn-Val-Glu-(Gln) of fragment#49.

Duplicate filters were prepared from the primary plating of the cDNA library and each set of filters were then hybridized using polynucleotide kinase $^{32}P$ labelled ("Molecular Cloning" Ed. Sambrook et al. 1989) oligonucleotides. The initial screening was carried out using the #1853 probe. Hybridization was carried out at 45°C in 6 x SSC, 5 x Denhardt's and 0.5% SDS for 4 hours. Filters were washed briefly at 45°C. Twenty clones were identified and picked as potential GPL cDNA clones. A secondary

screening using oligonucleotides #1853 as well as #1854 as probes identified a total of four cDNA clones as putative GPL clone by the criteria of showing positive hybridization to both probes. Plasmid DNA was prepared from each of the putative GPL clones and subjected to DNA sequencing by the Sanger dideoxy-termination-method (Sanger et al. PNAS (1977) 74, 5463-5467). Sequencing identified all four clones as true GPL clones, three of these being full-length cDNA clones of independent origin (i.e. the sequence of the cDNA inserts diverge at the very ends). The identity of cDNA clones was established on the basis of the peptide sequence resulting from microsequencing of the purified protein and on the basis of showing a high degree of homology to other known mammalian pancreatic lipases with the one major exception of a distinct discrepancy in the central part of the gene. This region encompasses the "loop structure" of the human pancreatic lipase. The loop structure of the human pancreatic lipase (HPL) forms what appears from X-ray crystallography to constitute a separate structural domain, delineated by two cystein residues (Winkler et al. (1990) Nature 343, 771-774). The discrepancy in the sequence of the guinea pig pancreatic lipase (GPL) relative to that of the human HPL protein corresponds precisely to a substitution of this 23 amino acid loop structure by a short 5 amino acid "mini-loop".

One of the GPL clones designated GPL#1, was sequenced in its entire length. The cDNA insert of GPL#1 is 1424 bp long encoding an open reading frame of 452 amino acids, 18 of which corresponds to a signal peptide. All four GPL cDNA clones were sequenced across the central homology-gap-region (the "mini-loop" region) and demonstrated to be identical.

Example 4

Isolation of a cDNA clone encoding a human pancreatic lipase (HPL):

A cDNA clone coding for the human pancreatic lipase (HPL) was obtained by PCR technology based on the sequence of the mature

HPL (i.e. minus the signal peptide) published by Winkler et al. (1990) Nature 343, 771-774. Starting from 3 µg of human placenta mRNA, a first strand cDNA was synthesized using conditions specified by the manufacturer of the Superscript reverse transcriptase (BRL). The reaction was carried out at 42°C for 45 min. Subsequently a tenth of this material was used as a template in a PCR reaction using the following oligonucleotides as primers:

AH#25:
CCTGGATCCGCCATG AA(AG) GA(AG) GT(ATGC) TA(TC) GA
(degeneracy = 64)

including the sequence corresponding to
Lys-Glu-Val-Cys-Thy-(Glu)
of the six most N-terminal amino acids of the mature HPL protein,
and

AH#26:
CCAGGATCCTCA AG(AG)CA (ATGC)GG (ATGC)GT (ATGC)A(AG) (ATGC)GT
(Degeneracy = 1024)

including the sequence corresponding to
Cys-Pro-Thr-Leu-Thr
of the five most C-terminal amino acids of HPL:

The conditions of the PCR reaction were as follows: a 100 µl reaction volume of buffer composition as recommended by the manufacturer (Perkin-Elmer Cetus AmpliTaq Kit) contained first strand material, dNTPs to a final concentration of 200 µM and 100 pmoles of each oligonucleotide primer. The reaction was run for 30 cycles at temperature levels of 98°C, 55°C and 72°C respectively. The resulting PCR fragment of approximately 1400 bp was subsequently subcloned as a BamHI fragment into the pBSKII vector. A clone resulting from this experiment, designated HPL#1, was shown by sequencing to encode a 431 amino acid protein that matched the published sequence of HPL. The DNA

sequence and derived amino acid sequence is shown in the appended Sequence Listing ID No. 3.

Example 5

Construction of human pancreatic lipase mutant (HPL(-)) with no loop structure:

The HPL mutant designated HPL(-) carries an internal deletion relative to the native form which corresponds exactly to the region non-homologous to the guinea pig enzyme. i.e. the loop structure. The details of the substitution are presented in the diagram of figure 1.

The mutant form of HPL was synthesized by PCR. First the two halves of the gene, the 5′ end and the 3′ end respectively, were synthesized in separate PCR reactions using the native HPL (HPL#1) gene as a template and oligonucleotide primers as indicated in figure 1. Subsequently these two fragments were used as templates in a second PCR ligation reaction using the oligonucleotides corresponding to the most 5′ respectively 3′ end of the gene as primers. The resulting fragment was subsequently cloned into the pBSKII (Stratagene) vector and sequenced throughout. An internal NcoI-AflII fragment was finally exchanged with the corresponding NcoI-AflII fragment of the native HPL gene as present in the Aspergillus expression plasmid pHD414 described in the European Patent Application No. 91610022.5.

Example 6

Construction of guinea pig pancreatic lipase mutant (GPL(+) containing a loop structure:

In the GPL mutant designated GPL(+), amino acids 239 to 245 of the mature protein were replaced with a 23 amino acid sequence corresponding to amino acids 238 to 262 of the HPL protein. This

region encompasses the loop structure. The details of this substitution are outlined in figure 2.

The GPL(+) mutant was synthesized in two consecutive PCR reactions. Initially the 5′ and 3′ halves of the GPL(+) gene were synthesized in separate PCR reactions using the following combination of oligonucleotides (the sequence appears from figure 2):

5′half: AH#65 and AH#131 3′half: AH#130 and AH#66.

Subsequently, the 5′ half and the 3′ half of the GPL(+) gene were combined by PCR ligation using oligonucleotides AH#65 and AH#66 as primers. The HindIII-SalI fragment resulting from this reaction was subcloned into the pBSKII vector and verified by sequencing. Subsequently, the GPL (+) HindIII-SalI fragment was subcloned into the HindIII, SalI sites of the Aspergillus expression vector pHD414 (described in European Patent Application No. 91610022.5).

Example 7

Construction of a truncated guinea pig pancreatic lipase ($GPL_{1-319}$):

Partial proteolytic cleavage by chymotrypsin of the porcine pancreatic lipase has demonstrated an ester-hydrolysing activity as being present in the extreme C-terminal part of the protein, viz. 336-449. It was, however, not possible to obtain the N-terminal fragment of the lipase (1-335) in intact form so as to analyze the enzymatic properties of this part of the protein (cf. De Caro et al. (1986) Eur. J. Biochem. 158, 601-607). In view of the extensive homology exhibited by the mammalian pancreatic lipases, it was decided to synthesize a recombinant truncated form of the guinea pig pancreatic lipase, express it in Aspergillus and subsequently characterize the enzymatic behaviour of the truncated GPL protein. The truncated form of the GPL protein was designated $GPL_{1-319}$ as the phenylalanine

residue of this protein corresponding to the cleavage site in the porcine protein constitutes residue 319 of the mature GPL protein.

The truncated form of the GPL was synthesized by PCR using the original GPL cDNA (GPL#1) as a template. The oligonucleotides AH#65 and AH#80 were used as primers for the PCR. The sequence of the oligonucleotides appears from figure 3. Oligonucleotide AH#65 corresponds to the 5′ end of the GPL gene and includes a SalI site. The oligonucleotide AH#80 corresponds to the GPL sequence including the codon for the phenylalanine residue number 319 immediately followed by a (premature) translational stop signal and a HindIII site.

The 1100 bp fragment resulting from the PCR reaction outlined above was subsequently cloned into pBSII using SalI-HindIII, checked by sequencing in its entity and finally cloned into the SalI-HindIII sites of the Aspergillus expression vector pHD414.

Example 8

Expression of guinea pig pancreatic lipase in **A. oryzae**.

An expression plasmid harbouring the guinea pig pancreatic lipase (GPL) gene was constructed from 3 elements, as outlined in Fig. 4. The synthetic oligonucleotide linker NOR 2460/2465 contains the N-terminal part of the GPL signal sequence to be connected to the TAKA promoter sequence at the BamH1 site. The BamH1-Bal1 linker sequence is as follows:

NOR2460/2465: 5′ GATCCGCTCGGCATGATGCTGTTT
3′ GCGAGCCGTACTACGACAAA

GCGTGGACCATCGGCCTTCTCCTGCTGG 3′ 52bp
CGCACCTGGTAGCCGGAAGAGGACGACC 5′ 48bp

The BalI-SalI fragment of ~1.5 kb from GPL#1 (cf. example 3) has the entire GPL sequence including the signal sequence. This

fragment was inserted into the BamHI-Xho1-cut expression vector pToC68 which is described in detail in International Patent Application No. PCT/DK91/00123. As controlling elements it has the TAKA amylase promoter from A.oryzae and the AMG terminator region from A.niger.

The GPL expression vector pHW713 was transformed into A.oryzae A1560-T40, a protease-deficient derivative of A.oryzae IFO 4177, using the procedure described in EP 238 023. Selection on acetamide was performed by cotransformation with pToC 186 harbouring the amdS gene from A.nidulans as a 2.7 kb XbaI fragment (Corrick et al. (1987), Gene 53, 63-71) on a pUC 19 vector (Yannisch-Perron et al. (1985), Gene 33, 103-119). We used a modification of the amdS gene with two up-promoter mutations, amdI9 (Hynes et al. (1988), Mol. Cell. Biol. 8, 2589-2596) and amdI66 (Katz et al., (1990), Mol. Gen. Genet. 220, 373-376).

Transformants were grown in YPD medium (Sherman et al., Methods in Yeast Genetics, Cold Spring Harbor Laboratory, 1981) for 3-4 days and analysed for new protein species in the supernatant by SDS-PAGE. GPL appeared as a band with an apparent Mw of 48 kD. The calculated Mw of GPL is 47.7 kD. The expected level of esterase activity was found using p-nitrophenyl laurate as substrate.

Example 9

Purification of rGPL from an Aspergillus oryzae culture

The pH of a supernatant from a culture of an A. oryzae transformant according to example 8 was adjusted to 7 by adding NaOH. The supernatant was subsequently applied to an anion exchange HPLC column (Si 300 Polyol CM, available from Serva, FRG) and subjected to high performance liquid chromatography (HPLC) followed by step-wise elution with 50 mM morpholinopropane sulfonic acid (MOPS)/1M NaCl/25% isopropanol as the eluent and desalination of active fractions on a Sephadex G-25 (Pharmacia, Sweden) column by means of 10 mM ammonium bicarbonate. After freeze-drying, the active fractions were subjected to size exclusion chromatography on a TSK G2000 SWG HPLC column using a 50 mM MOPS, pH 7.2, buffer, followed by desalination of active fractions on a Sephadex G-25 column by means of 10 mM ammonium bicarbonate. The active fractions were pooled and freeze-dried.

In all purification steps, PMSF was added to inhibit the alcaline protease produced by A. oryzae, and dithiothreitol was added to keep the free -SH group of the lipase reduced.

Example 10

Expression of pancreatic lipases in a baculovirus system

It is contemplated that all relevant pancreatic lipase constructs of the HPL and GPL series may be expressed in a baculovirus system. In this system expression of the gene of interest is driven by the autographa californica multiple nuclear polyhedrosis virus (AcMNPV) polyhedrin promoter, which in nature is responsible for a very high level expression of the polyhedrin protein, comprising up to 50% of total cellular protein at the end of the lytic cycle (Doerfler, W., Bohm, P. Eds. 1986, The Molecular Biology of the Baculoviruses, Current Topics in Microbiology and Immunology, Vol 131, Springer-Verlag,

Berlin.) Initially the HPL/GPL genes are cloned into the transfer vector pVL941 (Pharmingen) into which appropriate restriction sites have been introduced. Alternatively, appropriate HPL/GPL gene inserts are produced by PCR-directed mutagenesis aimed at introducing restriction sites at 5′ and 3′ ends of the respective genes. Subsequently the transfer plasmid is cotransfected with linearized BaculoGoldTM baculovirus DNA (Pharmingen), and due to a lethal deletion residing in the baculovirus DNA which is complemented by the transfer plasmid, nearly all of the released viruses contain integrated cDNA. A plaque assay is subsequently performed substantially as described by Volkman, L.E. and Summers, M.D. (1975), "Nuclear pyhedrosis virus detection: relative capabilities of clones developed from *Trichoplusia ni* ovarian cell line TN-368 to serve as indicator cells in a plaque assay", J.Virol. **16**, 1630-1637, resulting in selection of a single clone of recombinant virus. Following amplification of the virus, Sf9 cells (American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852-1776 Accession number CRL1711) are infected at an infection multiplicity of 1 substantially in accordance with the procedures described by Burand, J.P., Summers, M.D. and Smith, G.E. (1980), "Transfection with baculovirus DNA". Approximately four days post-infection - at which time the concentration of recombinant protein peaks - medium is collected and the protein is purified by a procedure as described in example 9 for expression of HPL/GPL in Aspergillus.

Example 11

Characterization of rGPL

Specific activity

The specific activity of rGPL purified as described in example 9 above, was determined using tributyrin and olive oil, respectively, as the substrate.

The tributyrin assay was carried out as described in Novo Nordisk Analysis Methods #AF95/5 (available from Novo Nordisk A/S on request), using tributyrin emulsified in gum arabic as the substrate and pH-stat titration at pH 7.0 and 30°C. Unless otherwise indicated, 0.05 mM DTT (dithiothreitol) was added to the substrate immediately prior to the start of the reaction.

The olive oil assay was carried out by pH-stat titration at pH 9.0 and 30°C. The substrate used was a substrate powder consisting of PVC particles (Pevicon PE 712, available from Kema Nord) coated with olive oil to an oil content of 16.7% w/w. To 1 g of substrate powder was added 20.5 ml buffer (70 mM $Na_2SO_4$, 2.5 mM Tris) and heated to 30°C, after which the substrate powder was dispersed by means of ultrasound for 15 seconds. Unless otherwise indicated, 0.05 mM DTT was added immediately prior to the start of the reaction.

The specific activity of rGPL was determined to be as follows:

| | |
|---|---|
| Tributyrin assay: | 1630 LU/mg protein |
| Olive oil assay: | 12 μmol free fatty acid/min./mg protein |

It should be noted that the olive oil assay is carried out in the absence of $Ca^{2+}$, which accounts for the difference in specific activity determined in the tributyrin and olive oil assays.

When the kinetics of rGPL and Humicola lanuginosa lipase were compared in the olive oil assay, it was found that the activity of the H. lanuginosa lipase was greatly reduced after about 1 minute. This reduction in activity is assumed to be caused by accumulation of liberated fatty acids at the oil/water interface resulting in a conformational change from active to inactive. rGPL, on the other hand, appeared to tolerate a far higher concentration of fatty acids at the oil/water interface than the H. lanuginosa lipase (cf. Fig. 5).

pH and temperature optimum

When determined in the tributyrin assay, the pH optimum of the rGPL was determined to be about 8.0, and the temperature optimum about 30°C (cf. Figs. 6 and 7).

Activity in the presence of surfactants

The activity of rGPL was determined in the olive oil assay in the presence of the following surfactants:

LAS: linear alkyl benzene sulphonate (Nansa 1169/P)
AOS: α-olefinsulphonate ($C_{14-18}$)
AEOS: alcohol ethylene glycol ether sulphate ($C_{12-15}$,3 EO; Dobanol 25-3S/60
AEO: alcohol ethoxylate ($C_{12-15}$,7 EO; Dobanol 25-7)
Alkyl phosphate (Berol 522)

It appears from Fig. 8 that rGPL is completely inhibited at a given concentration of the surfactant in question. It further appears that one surfactant, alkyl phosphate, results in a significantly lower degree of inhibition than the other surfactants studied. At the surfactant concentration usually employed in detergents (about 0.1-1 g/l), rGPL retains an activity between 40 and 100%.

Stability in the presence of detergents

The stability of rGPL was determined in model detergent systems by means of the tributyrin assay. 3 µg/ml rGPL was incubated in 1 ml detergent solution at 30°C for 20 minutes at pH 9.0 in the absence of DTT and at a water hardness of 0°dH. Samples were taken at t=0.5 and t=20 minutes. The activities shown in Table 3 below are given as the activity at t=20 relative to the activity at t=0.5.

Table 3

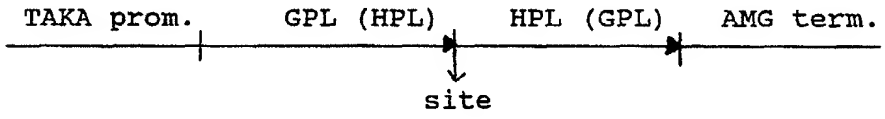
| | | M | N | P | Q | R | S | T | U | V | W |
|---|---|---|---|---|---|---|---|---|---|---|---|
| LAS | (g/l) | 0.1 | 1.0 | | | | | | | | |
| AOS | (g/l) | | | 0.1 | 0.1 | | | | | | |
| AEOS | (g/l) | | | | | 0.1 | 0.1 | | | | |
| alkyl phosphate | (g/l) | | | | | | | 0.1 | 0.1 | | |
| AEO | (g/l) | | | | | | | | | 0.1 | 0.1 |
| $Na_2SO_4$ | (g/l) | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| $Na_2CO_3$ | (g/l) | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 | 0.45 |
| Na metasilicate | (g/l) | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 |
| $Na_5P_3O_{10}H$ | (g/l) | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 | 1.25 |
| Activity (%) | | 0 | 0 | 14 | 0 | 93 | 71 | 102 | 104 | 100 | 72 |

It appears from the table that LAS and AOS are strongly inactivating, while AEOS and AEO are weakly inactivating at high concentrations (1 g/l), and alkyl phosphate does not have any inactivating effect in the model systems.

Example 12

Isolation of *in vivo* Recombined Hybrids of GPL and HPL

The HPL and GPL genes are so homologous that they will recombine to some extent in vivo, if they are placed as neighbours in a DNA construct and transformed into E. coli. The following arrangement in the Aspergillus expression vector pHD 414:




provides for analysis of hybrids from E. coli on the DNA level after cleavage at the unique restriction site between the two genes and re-transformation in E. coli to eliminate the non-recombined majority of plasmids. Potentially interesting hybrids are then transformed into A. oryzae for expression and analysis of enzyme properties by a transformation method known in the art, e.g. using the procedure described in Example 8 above.

Example 13

Isolation of *in vitro* Recombined Hybrid of GPL and HPL

The tandem configuration of GPL and HPL genes described in example 12 may form the basis for creating specific hybrids of the two genes in vitro. Alternatively, separate HPL and GPL DNA sequences may be used for the construction of in vitro recombinants.

The procedure involves 2 cycles of PCR which are illustrated in Fig. 9. PCR fragments produced in the first cycle are purified before the second cycle.

The fusion products are cloned after cleavage at unique restriction sites at some distance from the primers 1 and 4, which can be used in all constructions, while primers 2 and 3 vary according to the wanted recombination site. The primers are oligonucleotides prepared on the basis of corresponding parts of the GPL and/or HPL DNA sequences.

It will be understood that primer 1 and primer 4, respectively, may also be situated outside the genes, e.g. in the promoter region and terminator region, respectively.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Novo Nordisk A/S

(ii) TITLE OF INVENTION: Mammalian Pancreatic Lipase and Variants thereof

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Novo Nordisk A/S
(B) STREET: Novo Alle
(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) ZIP: DK-2880

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Sørensen, Lise Abildgaard
(C) REFERENCE/DOCKET NUMBER: 3625.204-WO

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: +45 4444 8888
(B) TELEFAX: +45 4449 3256
(C) TELEX: 37304

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1384 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: guinea pig

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 17..1375

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CTCGAGTCAG CTCGGC ATG ATG CTG TTT GCG TGG ACC ATC GGC CTT CTC          49
                  Met Met Leu Phe Ala Trp Thr Ile Gly Leu Leu
                    1               5                  10

CTG CTG GCC ACT GTC AGA GGA GCG GAA GTC TGC TAT TCA CAC CTT GGC         97
Leu Leu Ala Thr Val Arg Gly Ala Glu Val Cys Tyr Ser His Leu Gly
            15                  20                  25

TGC TTC TCA GAT GAA AAA CCA TGG GCA GGA ACC TCT CAG CGA CCT ATA        145
Cys Phe Ser Asp Glu Lys Pro Trp Ala Gly Thr Ser Gln Arg Pro Ile
        30                  35                  40

AAG TCA CTT CCC TCG GAC CCC AAG AAG ATC AAC ACT AGG TTT CTT CTG        193
Lys Ser Leu Pro Ser Asp Pro Lys Lys Ile Asn Thr Arg Phe Leu Leu
    45                  50                  55

TAC ACA AAT GAA AAC CAG AAT AGC TAC CAA TTG ATC ACG GCC ACT GAC        241
Tyr Thr Asn Glu Asn Gln Asn Ser Tyr Gln Leu Ile Thr Ala Thr Asp
60                  65                  70                  75

ATA GCC ACC ATC AAG GCT TCC AAC TTC AAC CTG AAC CGC AAG ACA CGC        289
Ile Ala Thr Ile Lys Ala Ser Asn Phe Asn Leu Asn Arg Lys Thr Arg
                80                  85                  90

TTC ATT ATA CAT GGC TTC ACG GAC AGT GGA GAG AAC AGC TGG CTA TCA        337
Phe Ile Ile His Gly Phe Thr Asp Ser Gly Glu Asn Ser Trp Leu Ser
            95                  100                 105

GAC ATG TGC AAG AAT ATG TTC CAA GTG GAG AAG GTG AAC TGC ATC TGT        385
Asp Met Cys Lys Asn Met Phe Gln Val Glu Lys Val Asn Cys Ile Cys
        110                 115                 120

GTG GAC TGG AAG GGG GGA TCT AAG GCC CAA TAC AGC CAA GCG TCT CAG        433
Val Asp Trp Lys Gly Gly Ser Lys Ala Gln Tyr Ser Gln Ala Ser Gln
    125                 130                 135

AAT ATT CGT GTT GTG GGC GCA GAG GTC GCT TAC TTG GTG CAA GTG CTT        481
Asn Ile Arg Val Val Gly Ala Glu Val Ala Tyr Leu Val Gln Val Leu
140                 145                 150                 155

TCG ACG AGC CTG AAT TAC GCC CCT GAG AAT GTA CAC ATC ATC GGC CAC        529
Ser Thr Ser Leu Asn Tyr Ala Pro Glu Asn Val His Ile Ile Gly His
                160                 165                 170
```

```
AGT TTG GGA GCG CAC ACT GCC GGG GAG GCG GGC AAG AGG CTG AAT GGC      577
Ser Leu Gly Ala His Thr Ala Gly Glu Ala Gly Lys Arg Leu Asn Gly
            175                 180                 185

CTC GTG GGC AGG ATC ACA GGG CTG GAT CCA GCA GAG CCA TAC TTC CAG      625
Leu Val Gly Arg Ile Thr Gly Leu Asp Pro Ala Glu Pro Tyr Phe Gln
        190                 195                 200

GAC ACG CCC GAG GAG GTT CGG CTG GAT CCA TCT GAT GCC AAG TTT GTG      673
Asp Thr Pro Glu Glu Val Arg Leu Asp Pro Ser Asp Ala Lys Phe Val
    205                 210                 215

GAT GTG ATT CAC ACA GAT ATT TCC CCT ATA CTT CCC TCC TTG GGT TTT      721
Asp Val Ile His Thr Asp Ile Ser Pro Ile Leu Pro Ser Leu Gly Phe
220                 225                 230                 235

GGA ATG AGC CAA AAG GTG GGC CAT ATG GAT TTC TTT CCA AAT GGA GGA      769
Gly Met Ser Gln Lys Val Gly His Met Asp Phe Phe Pro Asn Gly Gly
                240                 245                 250

AAA GAC ATG CCT GGA TGT AAA ACA GGA ATC TCT TGC AAC CAC CAC CGG      817
Lys Asp Met Pro Gly Cys Lys Thr Gly Ile Ser Cys Asn His His Arg
            255                 260                 265

AGC ATC GAG TAC TAT CAC AGC AGC ATC CTC AAC CCC GAA GGC TTC CTG      865
Ser Ile Glu Tyr Tyr His Ser Ser Ile Leu Asn Pro Glu Gly Phe Leu
        270                 275                 280

GGC TAC CCC TGT GCC TCC TAC GAC GAG TTC CAG GAG AGT GGC TGT TTC      913
Gly Tyr Pro Cys Ala Ser Tyr Asp Glu Phe Gln Glu Ser Gly Cys Phe
    285                 290                 295

CCT TGT CCA GCT AAA GGA TGT CCA AAA ATG GGG CAC TTT GCA GAC CAA      961
Pro Cys Pro Ala Lys Gly Cys Pro Lys Met Gly His Phe Ala Asp Gln
300                 305                 310                 315

TAT CCG GGA AAA ACC AAT GCT GTG GAA CAA ACC TTT TTC CTA AAC ACA     1009
Tyr Pro Gly Lys Thr Asn Ala Val Glu Gln Thr Phe Phe Leu Asn Thr
                320                 325                 330

GGA GCG AGT GAT AAC TTT ACT CGT TGG AGG TAT AAG GTA ACT GTC ACA     1057
Gly Ala Ser Asp Asn Phe Thr Arg Trp Arg Tyr Lys Val Thr Val Thr
            335                 340                 345

CTA TCT GGA GAA AAG GAT CCA AGC GGA AAC ATC AAT GTT GCT TTG TTG     1105
Leu Ser Gly Glu Lys Asp Pro Ser Gly Asn Ile Asn Val Ala Leu Leu
        350                 355                 360

GGA AAG AAC GGA AAC TCA GCA CAA TAT CAG GTT TTC AAG GGG ACC CTC     1153
Gly Lys Asn Gly Asn Ser Ala Gln Tyr Gln Val Phe Lys Gly Thr Leu
    365                 370                 375
```

```
AAA CCA GAT GCC AGT TAT ACT AAT AGC ATT GAT GTG GAG CTC AAT GTT      1201
Lys Pro Asp Ala Ser Tyr Thr Asn Ser Ile Asp Val Glu Leu Asn Val
380                 385                 390                 395

GGA ACA ATT CAG AAG GTT ACA TTC CTC TGG AAG AGA AGT GGG ATA AGT      1249
Gly Thr Ile Gln Lys Val Thr Phe Leu Trp Lys Arg Ser Gly Ile Ser
                400                 405                 410

GTC TCC AAG CCC AAA ATG GGG GCT TCC CGA ATC ACC GTG CAG AGC GGT      1297
Val Ser Lys Pro Lys Met Gly Ala Ser Arg Ile Thr Val Gln Ser Gly
            415                 420                 425

AAA GAT GGG ACC AAG TAT AAT TTT TGC AGC AGC GAC ATT GTG CAA GAA      1345
Lys Asp Gly Thr Lys Tyr Asn Phe Cys Ser Ser Asp Ile Val Gln Glu
        430                 435                 440

AAT GTT GAA CAG ACC CTT TCC CCT TGT TAATGACTCG AG                    1384
Asn Val Glu Gln Thr Leu Ser Pro Cys
    445                 450
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 452 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Met Leu Phe Ala Trp Thr Ile Gly Leu Leu Leu Ala Thr Val
 1               5                  10                  15

Arg Gly Ala Glu Val Cys Tyr Ser His Leu Gly Cys Phe Ser Asp Glu
            20                  25                  30

Lys Pro Trp Ala Gly Thr Ser Gln Arg Pro Ile Lys Ser Leu Pro Ser
        35                  40                  45

Asp Pro Lys Lys Ile Asn Thr Arg Phe Leu Leu Tyr Thr Asn Glu Asn
    50                  55                  60

Gln Asn Ser Tyr Gln Leu Ile Thr Ala Thr Asp Ile Ala Thr Ile Lys
65                  70                  75                  80

Ala Ser Asn Phe Asn Leu Asn Arg Lys Thr Arg Phe Ile Ile His Gly
                85                  90                  95

Phe Thr Asp Ser Gly Glu Asn Ser Trp Leu Ser Asp Met Cys Lys Asn
            100                 105                 110
```

Met Phe Gln Val Glu Lys Val Asn Cys Ile Cys Val Asp Trp Lys Gly
115 120 125

Gly Ser Lys Ala Gln Tyr Ser Gln Ala Ser Gln Asn Ile Arg Val Val
130 135 140

Gly Ala Glu Val Ala Tyr Leu Val Gln Val Leu Ser Thr Ser Leu Asn
145 150 155 160

Tyr Ala Pro Glu Asn Val His Ile Ile Gly His Ser Leu Gly Ala His
165 170 175

Thr Ala Gly Glu Ala Gly Lys Arg Leu Asn Gly Leu Val Gly Arg Ile
180 185 190

Thr Gly Leu Asp Pro Ala Glu Pro Tyr Phe Gln Asp Thr Pro Glu Glu
195 200 205

Val Arg Leu Asp Pro Ser Asp Ala Lys Phe Val Asp Val Ile His Thr
210 215 220

Asp Ile Ser Pro Ile Leu Pro Ser Leu Gly Phe Gly Met Ser Gln Lys
225 230 235 240

Val Gly His Met Asp Phe Phe Pro Asn Gly Gly Lys Asp Met Pro Gly
245 250 255

Cys Lys Thr Gly Ile Ser Cys Asn His His Arg Ser Ile Glu Tyr Tyr
260 265 270

His Ser Ser Ile Leu Asn Pro Glu Gly Phe Leu Gly Tyr Pro Cys Ala
275 280 285

Ser Tyr Asp Glu Phe Gln Glu Ser Gly Cys Phe Pro Cys Pro Ala Lys
290 295 300

Gly Cys Pro Lys Met Gly His Phe Ala Asp Gln Tyr Pro Gly Lys Thr
305 310 315 320

Asn Ala Val Glu Gln Thr Phe Phe Leu Asn Thr Gly Ala Ser Asp Asn
325 330 335

Phe Thr Arg Trp Arg Tyr Lys Val Thr Val Thr Leu Ser Gly Glu Lys
340 345 350

Asp Pro Ser Gly Asn Ile Asn Val Ala Leu Leu Gly Lys Asn Gly Asn
355 360 365

Ser Ala Gln Tyr Gln Val Phe Lys Gly Thr Leu Lys Pro Asp Ala Ser
370 375 380

```
Tyr Thr Asn Ser Ile Asp Val Glu Leu Asn Val Gly Thr Ile Gln Lys
385                 390                 395                 400

Val Thr Phe Leu Trp Lys Arg Ser Gly Ile Ser Val Ser Lys Pro Lys
                405                 410                 415

Met Gly Ala Ser Arg Ile Thr Val Gln Ser Gly Lys Asp Gly Thr Lys
            420                 425                 430

Tyr Asn Phe Cys Ser Ser Asp Ile Val Gln Glu Asn Val Glu Gln Thr
        435                 440                 445

Leu Ser Pro Cys
    450
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1404 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(A) ORGANISM: human

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 22..1371

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
TCTTGAACTA GTGGATCCGC C ATG AAG GAG GTG TGT TAC GAA AGA CTC GGC        51
                        Met Lys Glu Val Cys Tyr Glu Arg Leu Gly
                          1               5                  10

TGC TTC AGT GAT GAC TCC CCA TGG TCA GGA ATT ACG GAA AGA CCC CTC        99
Cys Phe Ser Asp Asp Ser Pro Trp Ser Gly Ile Thr Glu Arg Pro Leu
                15                  20                  25

CAT ATA TTG CCT TGG TCT CCA AAA GAT GTC AAC ACC CGC TTC CTC CTA       147
His Ile Leu Pro Trp Ser Pro Lys Asp Val Asn Thr Arg Phe Leu Leu
            30                  35                  40

TAT ACT AAT GAG AAC CCA AAC AAC TTT CAA GAA GTT GCC GCA GAT TCA       195
Tyr Thr Asn Glu Asn Pro Asn Asn Phe Gln Glu Val Ala Ala Asp Ser
        45                  50                  55
```

```
TCA AGC ATC AGT GGC TCC AAT TTC AAA ACA AAT AGA AAA ACT CGC TTT      243
Ser Ser Ile Ser Gly Ser Asn Phe Lys Thr Asn Arg Lys Thr Arg Phe
     60                  65                  70

ATT ATT CAT GGA TTC ATA GAC AAG GGA GAA GAA AAC TGG CTG GCC AAT      291
Ile Ile His Gly Phe Ile Asp Lys Gly Glu Glu Asn Trp Leu Ala Asn
 75                  80                  85                  90

GTG TGC AAG AAT CTG TTC AAG GTG GAA AGT GTG AAC TGT ATC TGT GTG      339
Val Cys Lys Asn Leu Phe Lys Val Glu Ser Val Asn Cys Ile Cys Val
                95                 100                 105

GAC TGG AAA GGT GGC TCC CGA ACT GGA TAC ACA CAA GCC TCG CAG AAC      387
Asp Trp Lys Gly Gly Ser Arg Thr Gly Tyr Thr Gln Ala Ser Gln Asn
            110                 115                 120

ATC AGG ATC GTG GGA GCA GAA GTG GCA TAT TTT GTT GAA TTT CTT CAG      435
Ile Arg Ile Val Gly Ala Glu Val Ala Tyr Phe Val Glu Phe Leu Gln
        125                 130                 135

TCG GCG TTC GGT TAC TCA CCT TCC AAC GTG CAT GTC ATT GGC CAC AGC      483
Ser Ala Phe Gly Tyr Ser Pro Ser Asn Val His Val Ile Gly His Ser
    140                 145                 150

CTG GGT GCC CAC GCT GCT GGG GAG GCT GGA AGG AGA ACC AAT GGG ACC      531
Leu Gly Ala His Ala Ala Gly Glu Ala Gly Arg Arg Thr Asn Gly Thr
155                 160                 165                 170

ATT GGA CGC ATC ACA GGG TTG GAC CCA GCA GAA CCT TGC TTT CAG GGC      579
Ile Gly Arg Ile Thr Gly Leu Asp Pro Ala Glu Pro Cys Phe Gln Gly
                175                 180                 185

ACA CCT GAA TTA GTC CGA TTG GAC CCC AGC GAT GCC AAA TTT GTG GAT      627
Thr Pro Glu Leu Val Arg Leu Asp Pro Ser Asp Ala Lys Phe Val Asp
            190                 195                 200

GTA ATT CAC ACG GAT GGT GCC CCC ATA GTC CCC AAT TTG GGG TTT GGA      675
Val Ile His Thr Asp Gly Ala Pro Ile Val Pro Asn Leu Gly Phe Gly
        205                 210                 215

ATG AGC CAA GTC GTG GGC CAC CTA GAT TTC TTT CCA AAT GGA GGA GTG      723
Met Ser Gln Val Val Gly His Leu Asp Phe Phe Pro Asn Gly Gly Val
    220                 225                 230

GAA ATG CCT GGA TGT AAA AAG AAC ATT CTC TCT CAG ATT GTG GAC ATA      771
Glu Met Pro Gly Cys Lys Lys Asn Ile Leu Ser Gln Ile Val Asp Ile
235                 240                 245                 250

GAC GGA ATC TGG GAA GGG ACT CGA GAC TTT GCG GCC TGT AAT CAC TTA      819
Asp Gly Ile Trp Glu Gly Thr Arg Asp Phe Ala Ala Cys Asn His Leu
                255                 260                 265
```

```
AGA AGC TAC AAA TAT TAC ACT GAT AGC ATC GTC AAC CCT GAT GGC TTT      867
Arg Ser Tyr Lys Tyr Tyr Thr Asp Ser Ile Val Asn Pro Asp Gly Phe
            270                 275                 280

GCT GGA TTC CCC TGT GCC TCT TAC AAC GTC TTC ACT GCA AAC AAG TGT      915
Ala Gly Phe Pro Cys Ala Ser Tyr Asn Val Phe Thr Ala Asn Lys Cys
        285                 290                 295

TTC CCT TGT CCA AGT GGA GGC TGC CCA CAG ATG GGT CAC TAT GCT GAT      963
Phe Pro Cys Pro Ser Gly Gly Cys Pro Gln Met Gly His Tyr Ala Asp
    300                 305                 310

AGA TAT CCT GGG AAA ACA AAT GAT GTG GGC CAG AAA TTT TAT CTA GAC     1011
Arg Tyr Pro Gly Lys Thr Asn Asp Val Gly Gln Lys Phe Tyr Leu Asp
315                 320                 325                 330

ACT GGT GAT GCC AGT AAT TTT GCA CGT TGG AGG TAT AAG GTA TCT GTC     1059
Thr Gly Asp Ala Ser Asn Phe Ala Arg Trp Arg Tyr Lys Val Ser Val
                335                 340                 345

ACA CTG TCT GGA AAA AAG GTT ACA GGA CAC ATA CTA GTT TCT TTG TTC     1107
Thr Leu Ser Gly Lys Lys Val Thr Gly His Ile Leu Val Ser Leu Phe
            350                 355                 360

GGA AAT AAA GGA AAC TCT AAG CAG TAT GAA ATT TTC AAG GGC ACT CTC     1155
Gly Asn Lys Gly Asn Ser Lys Gln Tyr Glu Ile Phe Lys Gly Thr Leu
        365                 370                 375

AAA CCA GAT AGT ACT CAT TCC AAT GAA TTT GAC TCA GAT GTG GAT GTT     1203
Lys Pro Asp Ser Thr His Ser Asn Glu Phe Asp Ser Asp Val Asp Val
    380                 385                 390

GGG GAC TTG CAG ATG GTT AAA TTT ATT TGG TAT AAC AAT GTG ATC AAC     1251
Gly Asp Leu Gln Met Val Lys Phe Ile Trp Tyr Asn Asn Val Ile Asn
395                 400                 405                 410

CCA ACT TTA CCT AGA GTG GGA GCA TCC AAG ATT ATA GTG GAG ACA AAT     1299
Pro Thr Leu Pro Arg Val Gly Ala Ser Lys Ile Ile Val Glu Thr Asn
                415                 420                 425

GTT GGA AAA CAG TTC AAC TTC TGT AGT CCA GAA ACC GTC AGG GAG GAA     1347
Val Gly Lys Gln Phe Asn Phe Cys Ser Pro Glu Thr Val Arg Glu Glu
            430                 435                 440

GTT CTG CTC ACG CTG ACA CCC TGC TGAGGATCCC CCGGGCTGCA GGAATTCGAT    1401
Val Leu Leu Thr Leu Thr Pro Cys
        445                 450

ATC                                                                 1404
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 450 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Lys Glu Val Cys Tyr Glu Arg Leu Gly Cys Phe Ser Asp Asp Ser
1 5 10 15

Pro Trp Ser Gly Ile Thr Glu Arg Pro Leu His Ile Leu Pro Trp Ser
20 25 30

Pro Lys Asp Val Asn Thr Arg Phe Leu Leu Tyr Thr Asn Glu Asn Pro
35 40 45

Asn Asn Phe Gln Glu Val Ala Ala Asp Ser Ser Ser Ile Ser Gly Ser
50 55 60

Asn Phe Lys Thr Asn Arg Lys Thr Arg Phe Ile Ile His Gly Phe Ile
65 70 75 80

Asp Lys Gly Glu Glu Asn Trp Leu Ala Asn Val Cys Lys Asn Leu Phe
85 90 95

Lys Val Glu Ser Val Asn Cys Ile Cys Val Asp Trp Lys Gly Gly Ser
100 105 110

Arg Thr Gly Tyr Thr Gln Ala Ser Gln Asn Ile Arg Ile Val Gly Ala
115 120 125

Glu Val Ala Tyr Phe Val Glu Phe Leu Gln Ser Ala Phe Gly Tyr Ser
130 135 140

Pro Ser Asn Val His Val Ile Gly His Ser Leu Gly Ala His Ala Ala
145 150 155 160

Gly Glu Ala Gly Arg Arg Thr Asn Gly Thr Ile Gly Arg Ile Thr Gly
165 170 175

Leu Asp Pro Ala Glu Pro Cys Phe Gln Gly Thr Pro Glu Leu Val Arg
180 185 190

Leu Asp Pro Ser Asp Ala Lys Phe Val Asp Val Ile His Thr Asp Gly
195 200 205

Ala Pro Ile Val Pro Asn Leu Gly Phe Gly Met Ser Gln Val Val Gly
210 215 220

His Leu Asp Phe Phe Pro Asn Gly Gly Val Glu Met Pro Gly Cys Lys
225 230 235 240

Lys Asn Ile Leu Ser Gln Ile Val Asp Ile Asp Gly Ile Trp Glu Gly
245 250 255

Thr Arg Asp Phe Ala Ala Cys Asn His Leu Arg Ser Tyr Lys Tyr Tyr
260 265 270

Thr Asp Ser Ile Val Asn Pro Asp Gly Phe Ala Gly Phe Pro Cys Ala
275 280 285

Ser Tyr Asn Val Phe Thr Ala Asn Lys Cys Phe Pro Cys Pro Ser Gly
290 295 300

Gly Cys Pro Gln Met Gly His Tyr Ala Asp Arg Tyr Pro Gly Lys Thr
305 310 315 320

Asn Asp Val Gly Gln Lys Phe Tyr Leu Asp Thr Gly Asp Ala Ser Asn
325 330 335

Phe Ala Arg Trp Arg Tyr Lys Val Ser Val Thr Leu Ser Gly Lys Lys
340 345 350

Val Thr Gly His Ile Leu Val Ser Leu Phe Gly Asn Lys Gly Asn Ser
355 360 365

Lys Gln Tyr Glu Ile Phe Lys Gly Thr Leu Lys Pro Asp Ser Thr His
370 375 380

Ser Asn Glu Phe Asp Ser Asp Val Asp Val Gly Asp Leu Gln Met Val
385 390 395 400

Lys Phe Ile Trp Tyr Asn Asn Val Ile Asn Pro Thr Leu Pro Arg Val
405 410 415

Gly Ala Ser Lys Ile Ile Val Glu Thr Asn Val Gl[illegible]ln Phe Asn
420 425 430

Phe Cys Ser Pro Glu Thr Val Arg Glu Glu Val Leu Leu Thr Leu Thr
435 440 445

Pro Cys
450

CLAIMS

1. A guinea pig pancreatic lipase (GPL) essentially free from other guinea pig proteins which has the amino acid sequence shown in the appended Sequence Listing ID No. 2, or an enzymatically active variant thereof.

2. A GPL according to claim 1, which is C-terminally truncated.

3. $GPL_{1-319}$ according to claim 2.

4. A GPL according to claim 1 or 2, which comprises an insertional substitution between amino acid 239 and amino acid 245 of the native GPL sequence.

5. A GPL according to claim 4, wherein the insertional substitution comprises the following amino acid sequence

Lys Ala Asn Leu
Gln Lys Asn Ile Leu Ser Gln Ile Val Asp Ile Asp Gly Ile Trp Glu

Ala
Gly Thr Arg Asp Phe Val Ala

or a subsequence thereof.

6. A GPL according to claim 5, wherein the insert comprises the amino acid sequence

Lys Lys Asn Ile Leu Ser Gln Ile Val Asp Ile Asp Gly Ile Trp Glu
Gly Thr Arg Asp Phe Ala Ala

7. A GPL according to any of claims 1-6, wherein $Cys_{103}$ or $Cys_{105}$ is replaced by another amino acid residue.

8. A GPL according to claim 7, wherein $Cys_{103}$ or $Cys_{105}$ is replaced by Thr or Ser.

9. A GPL according to any of claims 1-8, wherein $Ser_{154}$ is replaced by Thr and/or $Asp_{178}$ is replaced by Glu.

10. A recombinant DNA molecule comprising a DNA sequence encoding guinea pig pancreatic lipase (GPL) or an enzymatically active variant thereof.

11. A recombinant DNA molecule according to claim 10, wherein the DNA sequence is as shown in the appended Sequence Listing ID No. 1, or a modification of said sequence encoding GPL or an enzymatically active variant thereof.

12. A recombinant DNA molecule according to claim 10, wherein the DNA sequence encodes a C-terminally truncated GPL.

13. A recombinant DNA molecule according to claim 12, wherein the DNA sequence encodes $GPL_{1-319}$.

14. A recombinant DNA molecule according to claim 11, which comprises an insertional substitution between nucleotides 771 and 787 of the coding sequence of GPL.

15. A recombinant DNA molecule according to claim 14, wherein the insertional substitution encodes the following amino acid sequence

```
Lys          Ala                          Asn Leu
Gln Lys Asn Ile Leu Ser Gln Ile Val Asp Ile Asp Gly Ile Trp Glu

                        Ala
Gly Thr Arg Asp Phe Val Ala
```

or a fragment thereof.

16. A recombinant DNA molecule according to claim 15, wherein the insertional substitution comprises the following nucleotide sequence

AAA AAG AAC ATC TTG TCT CAA ATC GTT GAC ATC GAC GGT ATC TGG GAA GGT ACC AGA GAC TTC GCT GCT

17. A recombinant DNA molecule according to claim 11, which comprises a mutation in the codon specifying $Cys_{103}$ or $Cys_{105}$ so that it codes for another amino acid residue, in particular Ser or Thr.

18. A human pancreatic lipase (HPL) variant which is deleted of one or more amino acids in the sequence between $Cys_{238}$ and $Cys_{262}$.

19. A HPL variant according to claim 18, wherein the sequence between $Cys_{238}$ and $Cys_{262}$ is replaced by the sequence Lys-Thr-Gly-Ile-Ser.

20. A HPL variant comprising a C-terminal truncation.

21. $HPL_{1-336}$ according to claim 21.

22. A HPL variant according to claim 20 or 21, which is deleted of one or more amino acids in the sequence between $Cys_{238}$ and $Cys_{262}$.

23. A HPL variant wherein $Cys_{102}$ or $Cys_{104}$ is replaced by another amino acid residue.

24. A HPL variant according to claim 23, wherein $Cys_{102}$ or $Cys_{104}$ is replaced by Thr or Ser.

25. A HPL variant according to any of claims 18-24, wherein $Cys_{102}$ or $Cys_{104}$ is replaced by another amino acid residue.

26. A HPL variant according to claim 25, wherein $Cys_{102}$ or $Cys_{104}$ is replaced by Thr or Ser.

27. A HPL variant according to any of claims 18-26, wherein $Ser_{153}$ is replaced by Thr and/or $Asp_{177}$ is replaced by Glu.

28. An enzymatically active recombinant lipase variant which comprises at least one GPL fragment and at least one HPL fragment.

29. A DNA sequence encoding any of the lipase variants claimed in claims 19-27 or 28.

30. A process for the extracellular production of a mammalian pancreatic lipase in a filamentous fungus, the process comprising

(a) providing a recombinant DNA vector which comprises a DNA sequence encoding a mammalian pancreatic lipase and a DNA sequence encoding a preregion permitting secretion of the expressed mammalian pancreatic lipase into the culture medium,

(b) transforming a suitable filamentous fungus with the recombinant DNA vector of step (a), and

(c) culturing the transformed filamentous fungus in a suitable culture medium under conditions conducive to the production of the mammalian pancreatic lipase.

31. A process according to claim 30, wherein the filamentous fungus is a strain of Aspergillus sp.

32. A process according to claim 31, wherein the Aspergillus sp. is Aspergillus oryzae.

33. A process according to claim 30, wherein the preregion is derived from a gene encoding an Aspergillus sp. amylase or glucoamylase, a gene encoding a Rhizomucor miehei lipase, a gene encoding a Humicola cellulase or xylanase, or a gene encoding a mammalian pancreatic lipase.

34. A process according to claim 33, wherein the preregion is derived from the gene encoding A. oryzae TAKA amylase, A. niger neutral α-amylase, A. niger acid-stable α-amylase, A. niger glucoamylase, H. insolens cellulase or xylanase, or the gene encoding guinea pig, human, canine or porcine pancreatic lipase.

35. A process according to any of claims 30-34, wherein the mammalian pancreatic lipase is guinea pig pancreatic lipase or human pancreatic lipase.

36. A process according to claim 35, wherein the guinea pig pancreatic lipase is one encoded by a recombinant DNA molecule according to any of claims 10-17.

37. A process according to claim 35, wherein the human pancreatic lipase is a HPL variant according to any of claims 18-27 or a recombinant lipase variant according to claim 28.

38. A detergent additive comprising a GPL according to any of claims 1-9, a HPL variant according to any of claims 18-27 or a recombinant lipase variant according to claim 28, preferably in the form of a non-dusting granulate, stabilized, or protected enzyme.

39. A detergent additive according to claim 38 which contains 0.2-200 mg enzyme protein per gram of the additive.

40. A detergent additive according to claim 38, which further comprises another enzyme such as a protease, amylase, cellulase or peroxidase.

41. A detergent composition comprising a GPL according to any of claims 1-9, a HPL variant according to any of claims 18-27 or a recombinant lipase variant according to claim 28.

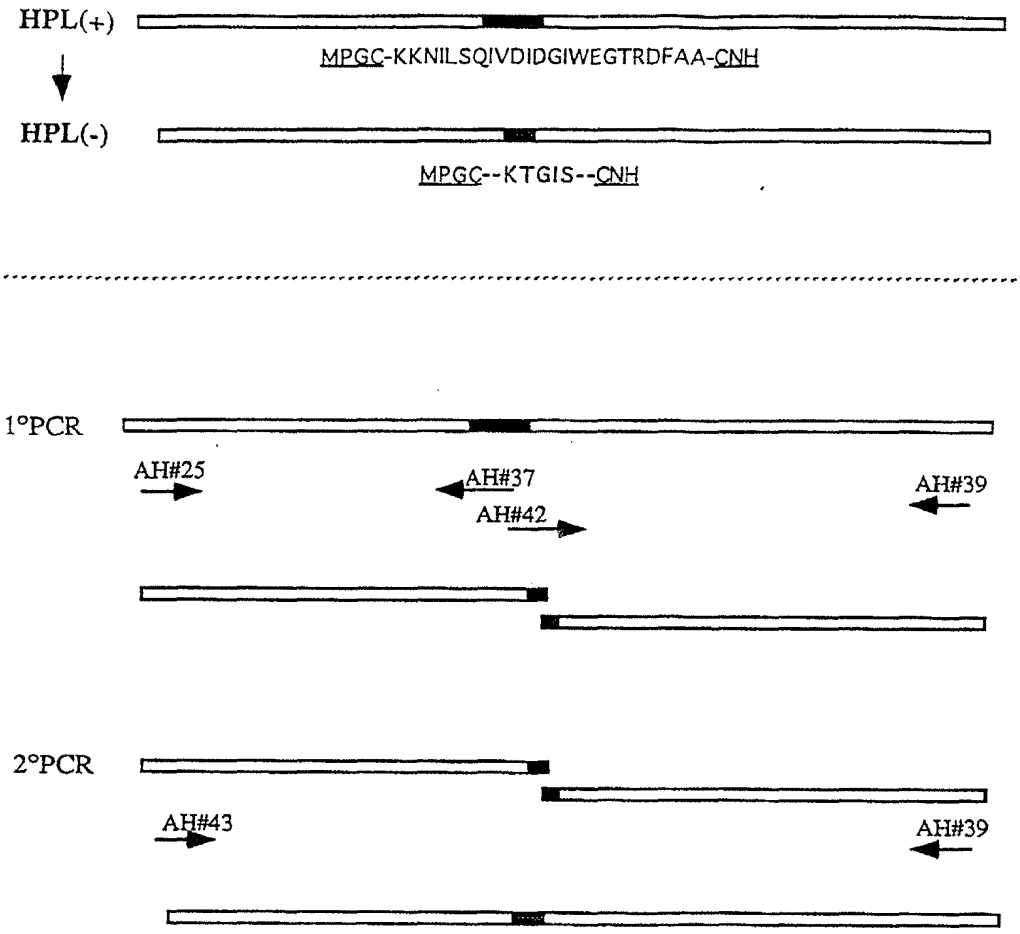
42. A detergent composition according to claim 41, which further comprises another enzyme such as a protease, amylase, cellulase or peroxidase.

43. A detergent composition according to claim 41 or 42, which as surfactant comprises an alkyl phosphate.

44. A GPL according to any of claims 1-9 for use as a digestive enzyme.

45. A HPL variant according to any of claims 18-27 for use as a digestive enzyme.

46. A recombinant lipase variant according to claim 28 for use as a digestive enzyme.




AH#25: CCT GGA TCC GCC ATG AA(AG) GA(AG) GT(ATGC) TG(TC) TA(TC) GA

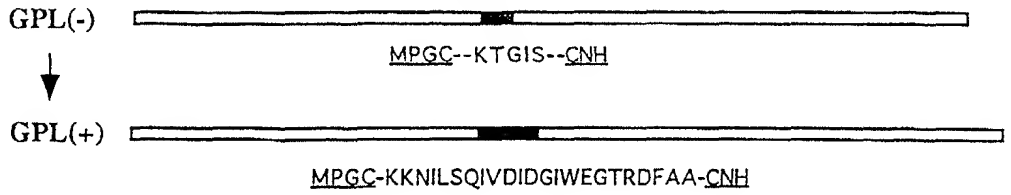
AH#37: ACA ACT AAT TCC AGT TTT ACA TCC TGG CAT TTC CAC

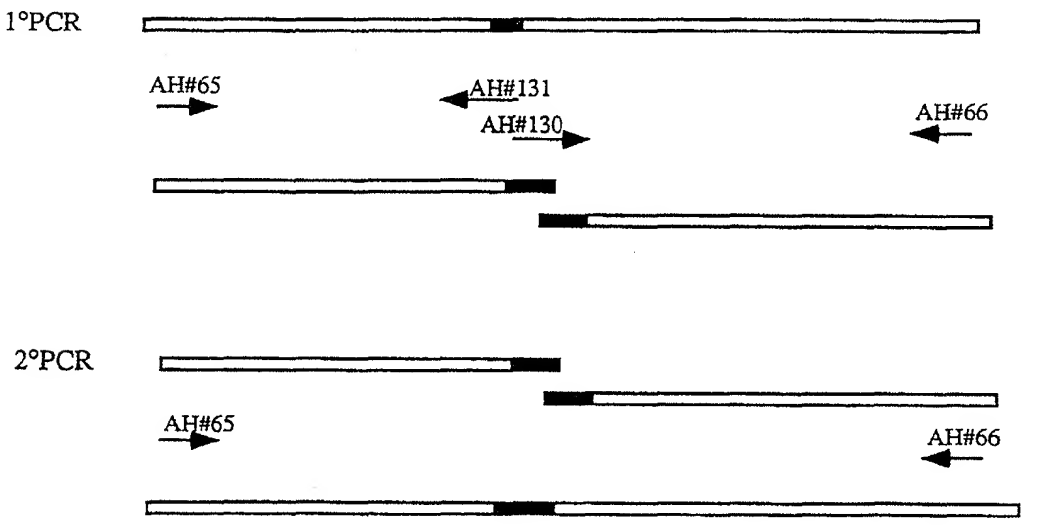
AH#39: CCA GGA TCC TCA GCA GGG TGT CAG

AH#42: TGT AAA ACT GGA ATT AGT TGT AAT CAC TTA AGA AGC

AH#43: CCT GGA TCC GCC ATG AAG GAG

Fig. 1




AH#65: GAAAGTCCGAGTCGACTCAGCTCGGCATGAT

AH#66: TTAGCAAGCCTAAGCTTTCATTAACAAGGGGA

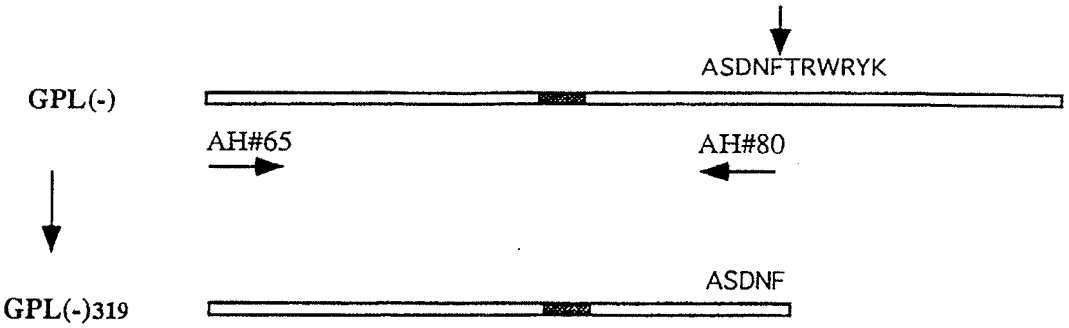
AH#131: CCAGATACCATCGATATCAACGATTTGAGA

CAAGATGTTCTTCTTACATCCAGGCATGTCTTTTCC

AH#130: GTTGATATCGATGGTATCTGGGAAGGTACCA

GAGACTTCGCTGCTTGCAACCACCACCGGAGCATC

Fig. 2




AH#65: GAAAGTCCGAGTCGACTCAGCTCGGCATGAT

AH#80: TTCCGAAGCTTAGATCTTCACTAAAAGTTATCACTCGCTCC

Fig. 3

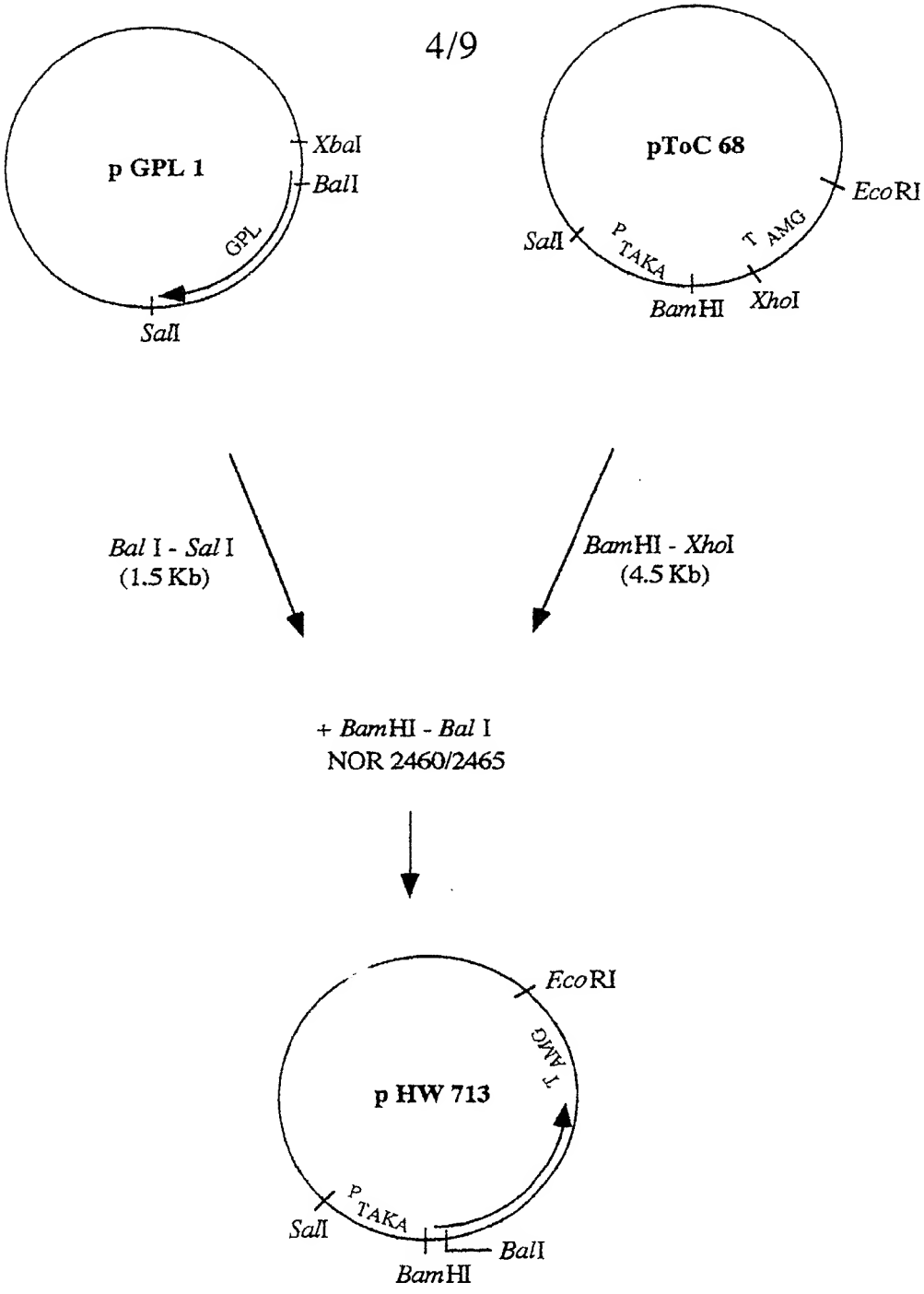

p GPL 1
XbaI
BalI
GPL
SalI
pToC 68
EcoRI
SalI
P TAKA
T AMG
BamHI
XhoI
Bal I - Sal I
(1.5 Kb)
BamHI - XhoI
(4.5 Kb)
+ BamHI - Bal I
NOR 2460/2465
p HW 713
EcoRI
T AMG
P TAKA
SalI
BalI
BamHI

Fig. 4

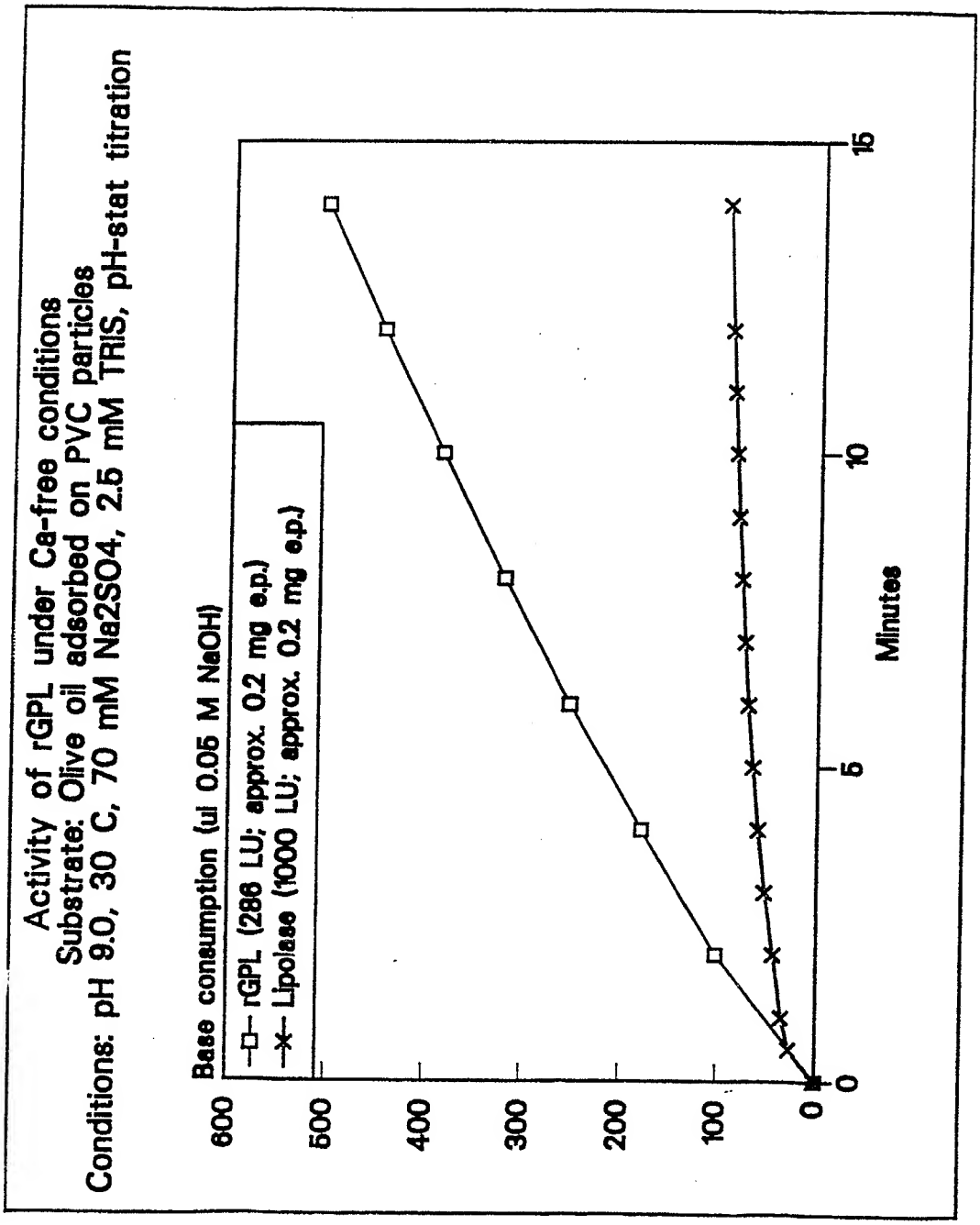

Activity of rGPL under Ca-free conditions
Substrate: Olive oil adsorbed on PVC particles
Conditions: pH 9.0, 30 C, 70 mM Na2SO4, 2.5 mM TRIS, pH-stat titration
Base consumption (ul 0.05 M NaOH)
rGPL (286 LU; approx. 0.2 mg e.p.)
Lipolase (1000 LU; approx. 0.2 mg e.p.)
600
500
400
300
200
100
0
0
5
10
15
Minutes

Fig. 5

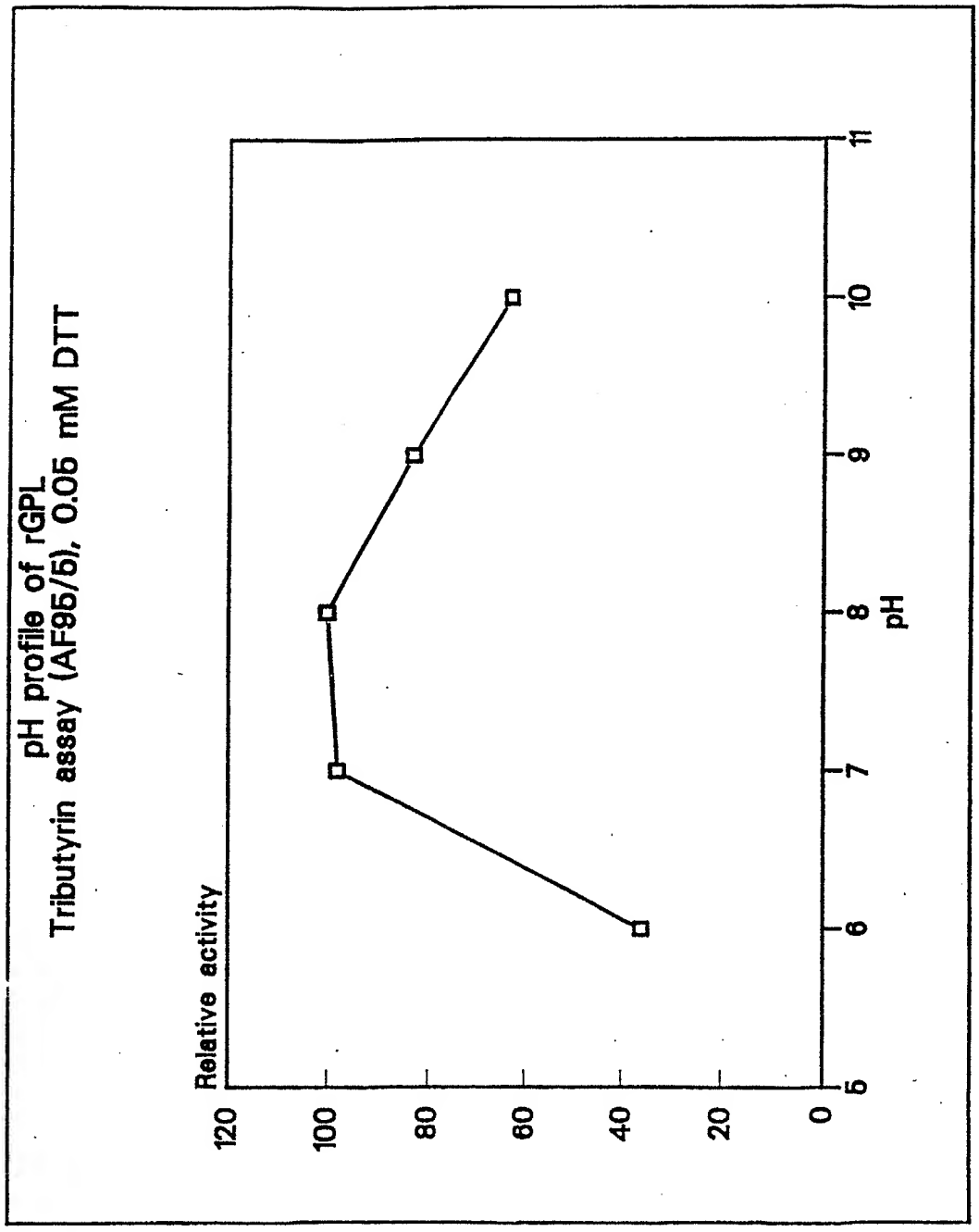

pH profile of rGPL
Tributyrin assay (AF95/6), 0.05 mM DTT
Relative activity
120
100
80
60
40
20
0
5
6
7
8
9
10
11
pH

Fig. 6

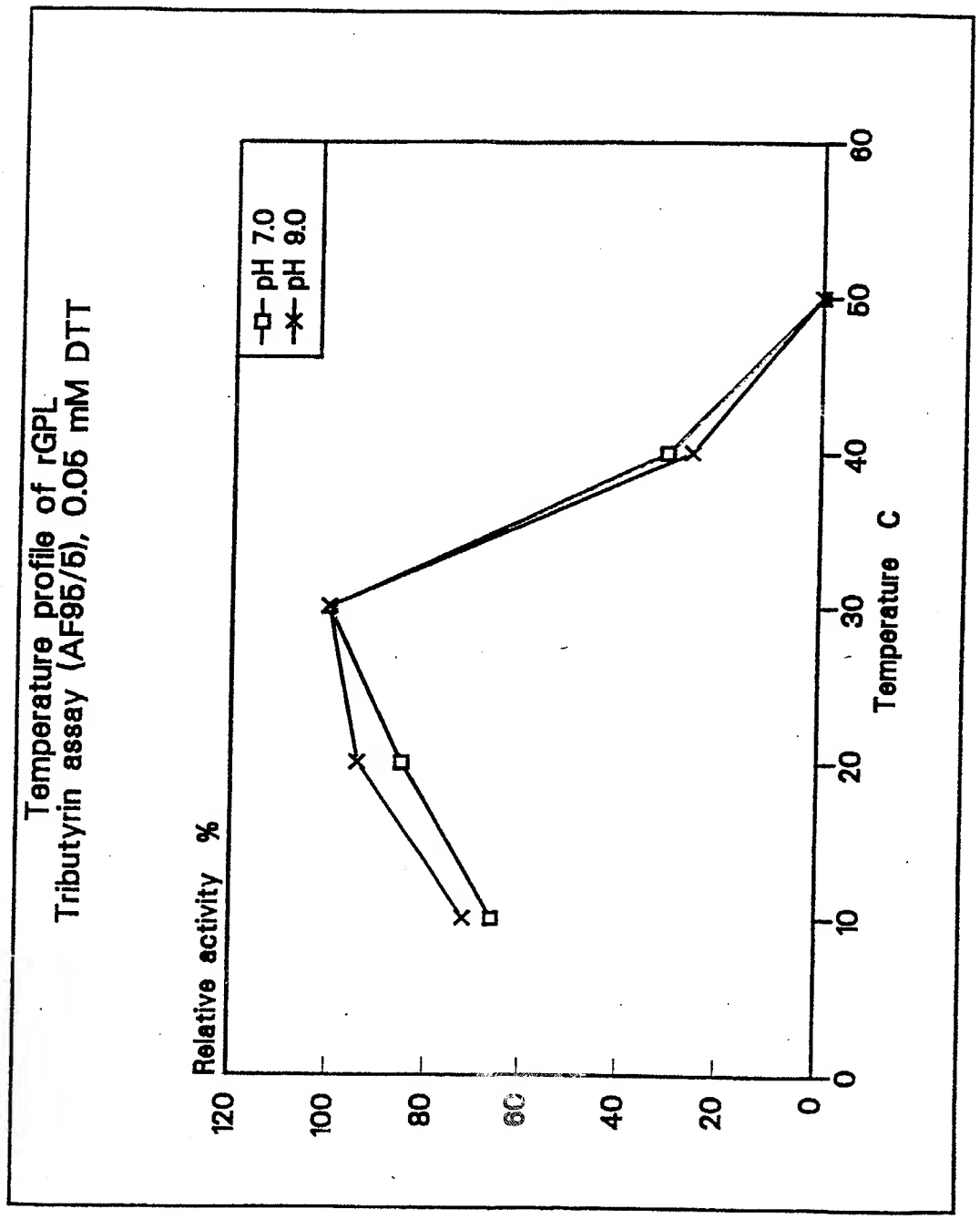

Temperature profile of rGPL
Tributyrin assay (AF95/6), 0.05 mM DTT
pH 7.0
pH 8.0
Relative activity %
120
100
80
60
40
20
0
0
10
20
30
40
50
60
Temperature C

Fig. 7

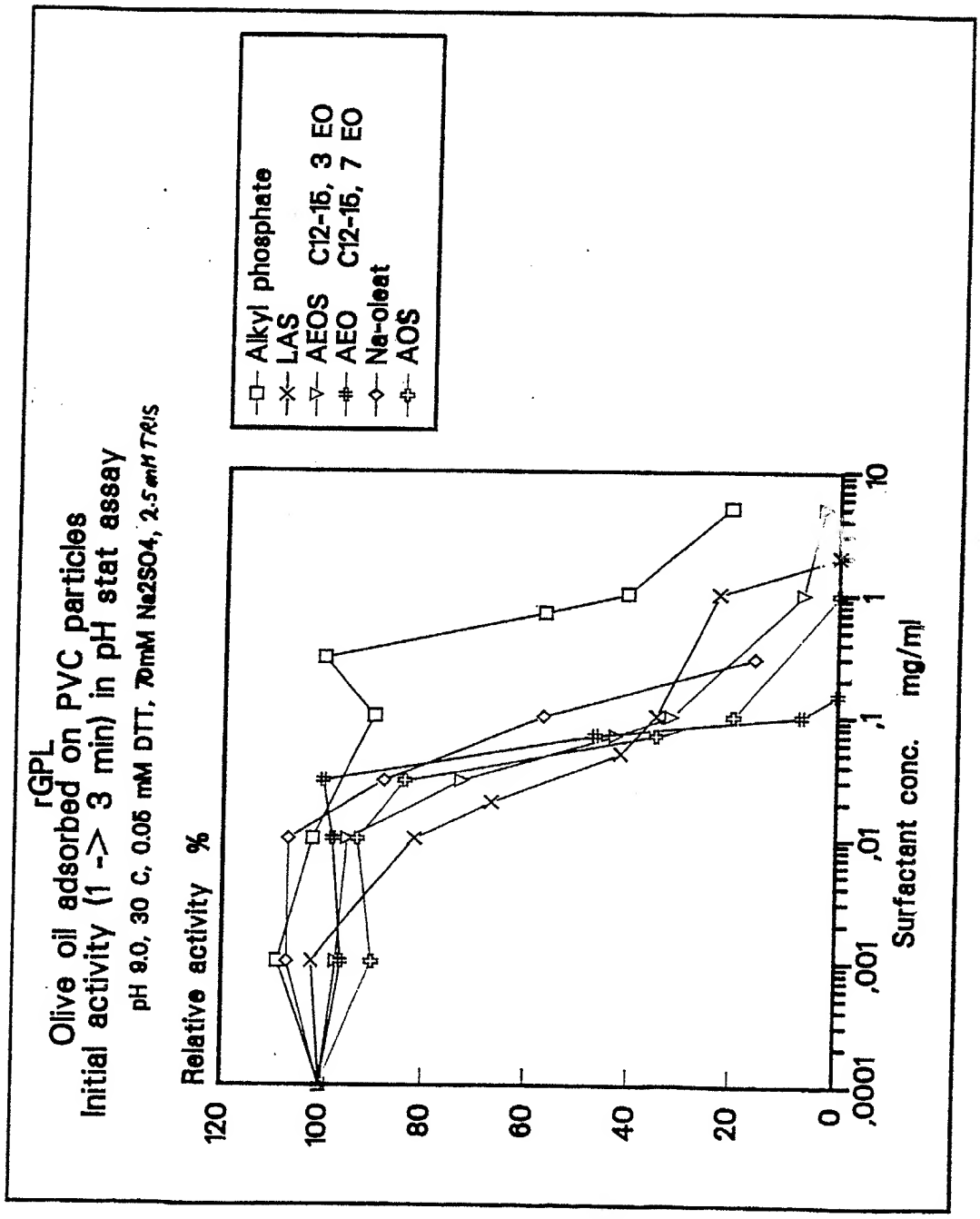

rGPL
Olive oil adsorbed on PVC particles
Initial activity (1 -> 3 min) in pH stat assay
pH 9.0, 30 C, 0.05 mM DTT, 70mM Na2SO4, 2.5 mM TRIS
Relative activity %
120
100
80
60
40
20
0
Surfactant conc. mg/ml
,0001
,001
,01
,1
1
10
Alkyl phosphate
LAS
AEOS C12-15, 3 EO
AEO C12-15, 7 EO
Na-oleat
AOS

Fig. 8

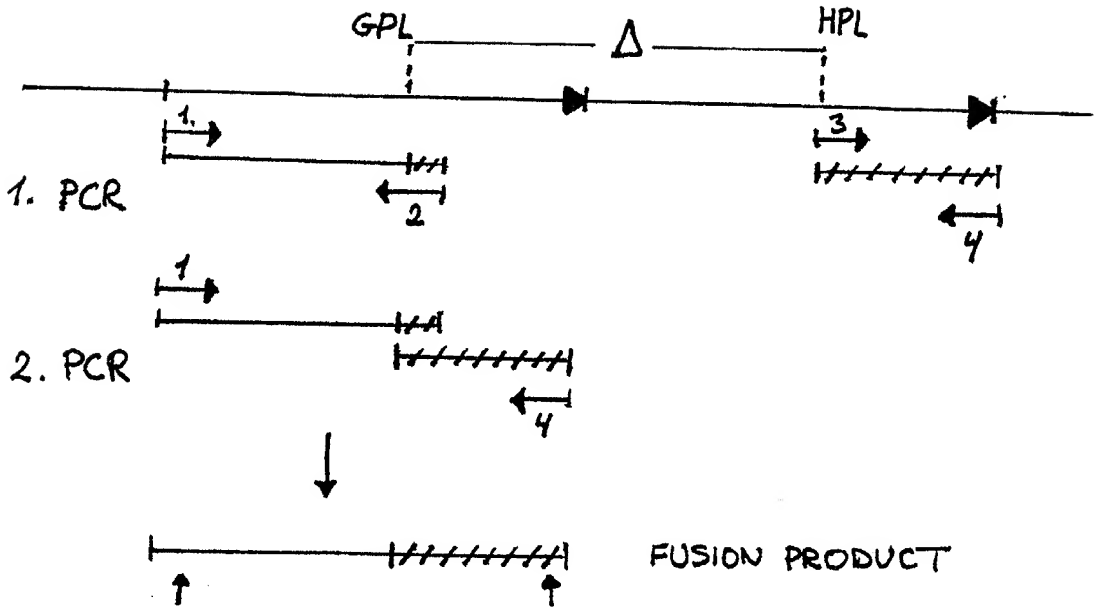



Fig. 9

# INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 92/00200

**I. CLASSIFICATION OF SUBJECT MATTER** (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 N 9/20, C 12 N 15/55//(C 12 R 1:69)

**II. FIELDS SEARCHED**

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| IPC5 | C 12 N |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in Fields Searched[8]

SE,DK,FI,NO classes as above

**III. DOCUMENTS CONSIDERED TO BE RELEVANT**[9]

| Category * | Citation of Document,[11] with indication, where appropriate, of the relevant passages[12] | Relevant to Claim No.[13] |
|---|---|---|
| P,X | WO, A1, 9205249 (NOVO NORDISK A/S) 2 April 1992, see page 8, line 36 - page 9, line 5; page 9, line 23 - line 27; page 10, line 17 - line 35; page 16, line 14 - page 17, line 31; claim 38 -- | 18-22, 28,29, 30-35, 37,38-41 |
| X | NATURE, Vol. 343, 1990 F.K. Winkler et al: "Structure of human pancreatic lipase", pp 771-774 | 20,29 |
| Y | | 30-35, 37 |
| A | -- | 1-17,30-44 |
| Y | EP, A2, 0238023 (NOVO INDUSTRI A/S) 23 September 1987, see page 3, line 40 - page 5, line 36; page 6, line 1 - line 6; claims 1-21 -- | 30-35, 37 |

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

**IV. CERTIFICATION**

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 5th November 1992 | 27 -11- 1992 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| SWEDISH PATENT OFFICE | Mikael G:son Bergstrand |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
|---|---|---|
| A | GENE, Vol. 58, 1987 S. Enerbäck et al: "Molecular cloning and sequence analysis of cDNA encoding lipoprotein lipase of guinea pig", pp 1-12 -- | 1-17-30-44 |
| A | Dialog Information Services, file 155: Medline, Dialog accession no. 05180960, Medline accession no. 84104960, Fauvel J. et al: "Substrate specificity of two cationic lipases with high phospholipase A1 activity purified from quines pig pancreas. I. Studies on neutral glycerides", & Biochim Biophys Acta Jan 17 1984, 792 (1) p 65-71 -- | 1-17,30-44 |
| A | Dialog Information Services, file 155: Medline, Dialog accession no. 05180961, Medline accession no. 84104961, Fauvel J et al: "Substrate specificity of two cationic lipases with high phospholipase A1 activity purified from quines pig pancreas. II. Studies on glycerophospholipides", Biochim Biophys Acta Jan 17 1984, 792 (1) p 72-8 -- | 1-17,30-44 |

---

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet!

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additonal fee.

Remark on Protest

☒ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional seach fees.

Claims 1-15 completely, 55-66 partially.

A lipase variant, where the electrostatic charge and/or the hydrophobicity of the lipid contact zone has been changed.

claims 16-37 completely, 55-66 partially.

A lipase variant, where the surface conformation of the lipid contactzone has been changed

claims 38-54 completely 55-66 partially.

A lipase variant supplied with a surface loop structure, which covers the active serine when the lipase is inactive, and which changes its conformation when the lipase is activated so as to make the active serine accessible to a lipid substrate.

These three categories of lipase variants are not considered have enough technical relationship so as to form a single inventive concept, particularly since it is known by EP,A2, 375 102 to change certain aminoacid residues of a lipase from Pseudomonas putida.

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. PCT/DK 92/00200

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the Swedish Patent Office EDP file on 30/09/92 The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A1- 9205249 | 92-04-02 | AU-D- | 8617291 | 92-04-15 |
| EP-A2- 0238023 | 87-09-23 | EP-A- | 0489718 | 92-06-10 |
| | | JP-A- | 62272988 | 87-11-27 |